



EFFECTS OF BENZO(A)PYRENE ON HUMAN BREAST CANCER CELL LINES

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ABSTRACT

Various epidemiological studies show that breast cancer is the most common type of cancer in women. Tobacco smoking is associated with increased risk of breast cancer. Polycyclic aromatic hydrocarbons (PAHs) are formed by the incomplete combustion of organic material and are present e.g. in tobacco smoke. Benzo(a)pyrene (BP) is one of the PAHs. BP is metabolized to Benzo(a)pyrene-diol-epoxide (BPDE) which is highly mutagenic and carcinogenic. The aim of this study was to elucidate carcinogenesis related cellular responses induced by BP exposure in breast cancer cell lines and analysing the protective role of p53 protein. Four different human breast cancer cell lines including one with wild type (wt) TP53 gene (ZR-75-1) and three (MDA-MB-231, MDA-MB-486 and T-47-D) with mutant TP53 gene were used for this purpose. Post-translational modifications (e.g. phosphorylations and acetylation) of p53 protein are indicators of activation of p53 protein. Cells were exposed to BP (1µM and 10µM for 48h) in duplicate fashion. The activation of p53 protein in all studied cancer cell lines was compared and evaluated at level of Ser-15 and Ser-392 phosphorylations. Ser-392 phosphorylation was not observed in MDA-MB-231 and T-47-D cell lines after BP exposure. It was found that BP increased phosphorylation of p53 at Ser-392 in ZR-75-1 cells, which have wt p53 protein and in MDA-MB-468 which have mutated p53 protein. Activation of p53 protein by BP is an indication of protective role of p53 against genotoxic insult aiming to protect from carcinogenesis.

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First praise is to Allah, the Almighty, on whom ultimately we depend for sustenance and guidance.

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Regards,

Muhammad Akram

DEDICATION

I would like to dedicate my thesis to my sweet mother

ABBREVIATIONS

4-ABP	4-aminobiphenyl
ADP	Adenosine Diphosphate
AKAP9	A-Kinase Anchor Protein 9
APS	Ammonium Persulfate
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia Mutated and RAD3 Related
Bax	Bcl-2-associated X protein
BDPE	Benzo(α)pyrene-7,8-diol-9,10-epoxide
BP	Benzo(a)Pyrene
BRCA	Breast Cancer Type 1 Susceptibility Protein
BRCA	Breast Cancer Type 2 Susceptibility Protein
BRIP1	Fanconi anemia group J protein
BSA	Bovine Serum Albumin
CAK	CDK Activating Kinase
CASP8	Caspase-8
CDH1	Cadherin-1
CDK	Cyclin Dependent Kinase
CHEK2	Serine / Threonine-Protein Kinase
Chk2 / FGFR2	Fibroblast Growth Factor Receptor 2
CK1	Casein kinase 1
CKII	Casein Kinase II
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA-PK	DNA dependent Protein Kinase
ds-DNAPK	Double Stranded DNA Dependent Protein Kinase
E3s	Ubiquitin Protein Ligases
ECL	Enhanced Chemiluminescence
EDTA	Ethylene Diamine Tetra Acetic acid
EGFR/HER/ERBB	Human Epidermal Growth factor Receptor
EMSA	Electrophoretic Mobility Shift Assay
ER	Estrogen Receptor
FBS	Foetal Bovine Serum

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish Peroxidase
IARC	International Agency for Research on Cancer
IDC-NST	Invasive Ductal Carcinomas of Special Type
JNK	C-JUN N-terminal Kinase
kD	Kilo Dalton
LSP1	Lymphocyte-Specific Protein 1
MAP3K1	Mitogen-Activated Protein Kinase Kinase Kinase 1
MDM2	Murine Double Minute 2
miRNA	Micro Ribose Nucleic Acids
mRNA	Messenger RNA
NaCl	Sodium Chloride
NAD	Nicotinamide Adenine dinucleotide
Na-pyruvate	Sodium Pyruvate
NNK	4-N-methyl-N-nitrosamino-1-(3pyridyl)-1-butanone
NP40	Nonidet-P40
PACs	Polycyclic Aromatic Compounds
PAHs	Polycyclic Aromatic Hydrocarbons
PARP	Poly ADP Ribose Polymerase
PBS	Phosphate Buffer Saline
PK	Protein Kinase
PR	Progesterone receptor
PUMA	p53 up-regulated modulator of apoptosis
PVDF	Polyvinylidene Fluoride
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RTKs	Receptor Tyrosine Kinases
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis
Ser	Serine
Thr	Threonine
TBS	Tris Buffer Saline
TBST	Tris Buffer Saline Tween

TEMED	N, N, N', N'-tetramethylethylenediamine
TNBC	Triple-Negative Breast Cancer
Tris-HCl	Tris-Hydrochloric Acid
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
Wt	Wild Type

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1 INTRODUCTION

Breast cancer is the most common cancer in women all over the world predominantly highest rate in US and Western Europe. It is the leading cause of death in their midlife (Smigal et al., 2006) Among Asia, Pakistan has highest incidence of breast cancer development, the causes of which is unknown. Breast cancer is the 3rd most common among all reported malignancies in women in Pakistan in last two years (Raza et al., 2012). According to Rudel et al (2003) environmental factors play an important role in the development of breast cancer. Strong evidence exists between incidence of breast cancer and exposure to polycyclic aromatic hydrocarbons (PAHs), organic solvents and polychlorinated biphenyls (Brody and Rudel, 2003). PAHs are produced as a result of incomplete combustion of organic material (like forest fires, traffic exhaust and tobacco smoking) from natural source. PAHs are present in atmosphere everywhere, so humans are exposed to PAHs all the time (Ravindra et al., 2008).

Benzo(a)pyrene (BP) is one of the polycyclic aromatic hydrocarbon produced. BP is ubiquitously present in environment (Xue and Warshawsky, 2005) .BP is also present in some foods in considerable quantities like in cooked red meat, fried chicken with skin and in fried eggs also (Ronco et al., 2011). BP is surrogate to all PAHs and is placed in Class-I by International Agency for Research on Cancer (IARC 2010). Metabolism of BP results in the formation of reactive metabolites. Best known of these metabolites is BP-7,8-diol-9,10-epoxide (BPDE) which is potential genotoxic and carcinogenic compound (Xue and Warshawsky, 2005). BPDE is very reactive and can bind to DNA resulting in formation of DNA adduct. As a consequence, tumor suppressor protein p53 is activated in those cells in which DNA adducts are formed. Activated p53 can cause cell cycle arrest and participate in DNA repair process. If DNA is irreparable, p53 can drive cells in apoptosis. (Huovinen et al., 2011, Tampio et al., 2008).

Caspases are the proteolytic enzymes which control the apoptotic pathway (Boatright and Salvesen, 2003). p53 has important role in the protection of organism against cancer growth e.g. by regulating apoptosis The apoptotic reactions induced by p53 protein are regulated in both nucleus and cytoplasm. The expression of pro-apoptotic genes like p53 up-regulated modulator of apoptosis (PUMA) and Bcl-2-associated X protein (Bax) is regulated in nucleus (Vousden and Lane, 2007). Apoptotic process in cytoplasm is regulated by interaction of p53

protein with both pro-apoptotic and anti-apoptotic proteins (Amaral Jd Fau - Xavier et al., 2010). The half-life of p53 protein is also increased from minutes to hours in those cells in which DNA is damaged (Fritsche et al., 1993).

The protective action of p53 is started by its post-translational modifications in response to DNA damage or cell stress. Common post-translational modifications of p53 protein are phosphorylations and acetylation of p53 protein (Lane and Levine, 2010). The tumour suppressor protein p53 is stabilized and activated via phosphorylation at various serine residues like Ser6, Ser15, Ser20, Ser37, Ser47 and Ser392. All these activation cascades act in different ways against cell stress. (Thompson et al., 2004). Ser15 and Ser20 phosphorylation of p53 are involved in inhibition of binding of murine double minute 2 (MDM2) protein to p53, this results in increased half-life of p53 protein (Bode and Dong, 2004). MDM2 protein is considered to be a negative regulator of p53 protein (Iwakuma and Lozano, 2003). Acetylation on C-terminal is the other way of p53 protein stabilization by inhibiting the recycling of p53 protein (Prives and Manley, 2001).

Tampio et al (2008) found an increased phosphorylation of p53 at ser¹⁵, ser²⁰ and ser⁴⁶ after exposure to BP in human MCF-7 breast cancer cells (Tampio et al., 2008). In this study, the effect of BP on phosphorylations of p53 at Ser-15 and Ser-392 in four other human breast cancer cell lines (MDA-MB-231, MDA-MB-468, T47-D and ZR-75-1) was studied.

2 LITERATURE REVIEW

2.1 Breast Cancer

Breast cancer is the major cause of death in women all over the world. Mortality rate to breast cancer is 18% among all the malignancies reported. Breast cancer screening at the age of 50-64 years revealed that more than 14,000 deaths in this age group in USA is because of incidence of breast cancer (McPherson et al., 2000). Mcpherson et al (2000) revealed that the mortality rate to breast cancer in women is highest in United States of America (USA) and lowest in Gambia (McPherson et al., 2000).

2.1.1 Pathogenesis of Breast Cancer

Breast cancer pathogenesis is stimulated when estrogen and progesterone bind to steroid receptors in epithelial cells of breast tissues. This binding promote cell growth, differentiation and survival of epithelial cells (Taneja et al., 2010). The other receptors which are present on the surface of epithelial cells are named as receptor tyrosine kinase (RTKs). These receptors also play an important role in the development of breast cancer via signal transduction (Pegram et al., 2000). Epidermal growth factor receptor / Human Epidermal Growth factor Receptor (EGFR / HER / ERBB) of RTKs family is especially involved in the development of breast cancer (Sergina et al., 2007).

Both estrogen and progesterone activate the nuclear receptors by acting as intracellular signaling molecules. These are hormones produced by adrenal glands and ovaries and they diffuse through cell membrane and then bind to the nuclear receptors. Once these hormones are bind to nuclear receptors a conformational change occurs which results into dimerization of estrogen receptors (ER) and progesterone receptors (PR) (Taneja et al., 2010). Growth factor receptor pathways and proto-oncogenes are directly modulated by the progesterone and estrogen (Taneja et al., 2010). EDGF/HER/ERBB family of RTKs is connected for the growth and development of breast tissue (Atalay et al., 2003). In most of the breast cancer cases ERBB family is overexpressed causing oncogenic signaling (Hynes and Lane, 2005). Strong evidence is present for involvement of EGFR, ERBB2, ERBB3 and ERBB4 for the pathogenesis of breast cancer (Atalay et al., 2003).

In 25-30% of breast cancer cases HER2/ERBB2 is over expressed (Slamon et al., 1989). HER2 is activated by homo-dimerization or hetero-dimerization with other EGFR family

members (Hynes and Lane, 2005). Hetero-dimerization of HER2-HER3 enhances mutagenesis in proliferating cells (Rowinsky, 2004). Overexpression and deregulated signaling of HER2 are associated with aggressive disease and is believed as negative prognostic factor in breast cancer (Hynes and Lane, 2005). HER2-HER3 heterodimer can activate various downstream targets, one of which is the PI3 kinase/ATK pathways (Figure 1) which is well known for its critical role in tumor-genesis (Hsieh and Moasser, 2007). HER4 can dimerize with all members of EGFR family. Upon activation of HER4 dimer various downstream signaling pathways are activated. The exact role of HER4 in the development of breast cancer is not fully known (Sartor et al., 2001).

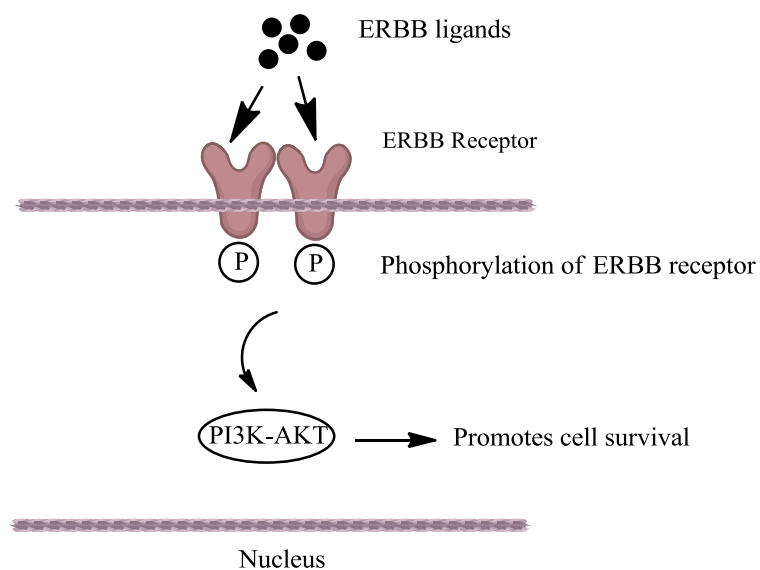


Figure 1. Activation of ERBB receptors with downstream signalling pathway: Phosphatidylinositol 3-kinase (PI3-AKT) pathway is activated upon interaction of p85 adaptor subunit of PI3K to the receptor. Modified from (Hynes and Lane, 2005)

2.1.2 Molecular Biology of Breast Cancer

Breast cancer has been considered as a single ailment with a common treatment protocol, however in reality it is a heterogeneous disease having at least four to five subtypes (Martín, 2006). Mavaddat et al (2010) conducted a study in which data from candidate gene studies was combined for assessing the relationship of gene with family history for development of cancer. The novel markers they used in their study are not fully genuine because it is unclear how they cause breast cancer. However, conducting such a big study shows that there are various histological and clinic-pathological subtypes of disease (Mavaddat et al., 2010).

How genetic variation is related to breast cancer pathology is the entire time burning topic for the researchers. High-throughput technologies are using global gene expression to find out the heterogeneity of breast cancer and have provided the new molecular subtypes. Five major intrinsic breast cancer subtypes have been identified like, HER2-enriched, Luminal A, Luminal B, Normal breast-like and a Basal like group. All these types of tumors give detailed knowledge on the initiation and progression of breast cancer, since these have very exciting clinical differences (Prat and Perou, 2011). Weigelt et al (2010) also describes the histological types of breast cancer like tubular, adenoid cystic, secretory, lobular and medullary carcinomas. These subtypes have not been studied in depth because of lack of sufficient data. Microarray analysis explains the expression profiling. It describes that histological types are more homogenous than invasive ductal carcinomas of special type (IDC-NST) (Weigelt et al., 2010).

A very good explanation of another type of breast cancer named Triple-Negative Breast Cancer (TNBC) was given by Podo et al in 2010. He said that TNBC is a form of breast cancer which lacks the gene for the expression of ER, PR and HER2. Phenotypically TNBC is very aggressive and responds partially to chemotherapy, so we can assume the poor prognosis of this type of cancer (Podo et al., 2010). Micro RNAs are small endogenous molecules which triggers and regulate e.g. the messenger RNA (mRNA) degradation or translation process. Le Quesne and Caldas, (2010) mentioned the up-regulated expression level of miRNA in breast cancer tissue in a study in which they analysed the miRNA biology in both normal breast tissues and breast cancer tissues (Le Quesne and Caldas, 2010). Hannafon et al (2011) described that up-regulated miR-21, miR10b and miR-27a act as oncogenes (Hannafon et al., 2011).

Kwei et al (2010) used DNA profiling technique for explaining the abnormalities of breast cancer at genomic level in three groups. Simpler group is known by mutation of whole chromosome arm, amplifier group is known by high-level DNA mutations at focal point and complex type is explained by low-amplitude changes. The mechanism of these changes at various DNA patterns is entangled by telomere non-functioning, genomic imbalance and improper repair of DNA molecules (Kwei et al., 2010).

2.1.3 Cancer Cell Lines as a Model for Breast Tumors

Human breast cancer cell lines are most widely used *in vitro* experimental model for studying breast tumors. Breast cancer cell lines are pure, genetically modified and are easy to culture. Breast cancer cell lines being a model for studying pathologies produce significant results when used for same protocol (Vargo-Gogola and Rosen, 2007). Other advantages of breast cancer cell lines as reported by Burdall et al (2003) include that they are easy to handle, they can be grown in infinite quantities because of unlimited self-replication, have high degree of homogeneity and can be replaced with frozen stocks if contaminated (Burdall et al., 2003). Human cell lines are being chosen over mice cell lines because same onco-gene may produce different phenotypes in different species (Rangarajan and Weinberg, 2003).

The irrelevancy of cell lines as experimental model is due to well explained common errors (Masters, 2000). These errors can be explained as follows, Genome instability: The constant instability might results into completely new cell line. It could be prevented by limiting the number of passages and to always come back to cell frozen during early passages (Hughes et al., 2007). Cross contamination is the other trivial error in cell lines. It was first described by Nelson-Rees et al (1970). They showed that most of cancer cell lines were Hela cells at that time. DNA fingerprinting technique can be used for their verification (Nelson-Rees et al., 1981). Culture condition is the 3rd trivial error, in this case for example extracellular matrix obliged on cells *In vitro* changed the expression of genes, their morphology and chromatin condensation (Le Beyec et al., 2007). The last but not least trivial error reported was infections typically by mycoplasma. Infections can completely change the cell culture properties. They can be easily diagnosed but not cured so easily (Harlin and Gajewski, 2008).

It was mentioned by Burdall et al (2003) that use of established cell lines should be continued as a model for breast cancer. It is considered as essential that researcher should be aware of limitations for use of cancer cell line while designing an experiment. It was also established that primary cell cultures provide more relevant model of the disease. It was further explained by Brudall et al (2003) that well managed planning and controlled experimentation with breast cancer cell lines may provide better understanding about pathology of breast cancer (Burdall et al., 2003).

Lacroix and Leclercq (2004) explained how cancer cells in a tumor are accurately represented by breast cancer cell lines (BCC). They mentioned that breast tumor is regarded

as sub-populations showing same phenotypes. Clonal expansion of only one sub-population is not regarded as involved in tumor development. Isolated BCC are regarded as representative of In vivo BCC. However changes in BCC may develop voluntarily or by use of chemicals. This might happen because of genetic instability. There are different types of carcinomas of breast depending on genotype/phenotype. The understanding of breast cancer biology can be enhanced by studying thoroughly the less investigated cell lines. Infrequent type breast cancer cell lines and those arise from metastatic sites also needs further research (Lacroix and Leclercq, 2004).

2.2 Chemical Carcinogenesis

The utilization of chemical compounds has many advantages in society. For example pesticides enable the production of foodstuffs in adequate quantities which help millions of people for to fulfill their needs. Besides advantages, chemical compounds have shortcomings in the form of toxic effects. These effects depend on exposure to the type of chemical compounds used. The range of their toxic effects varies from immediate death to a progressive process like chemical carcinogenesis. Initiation, promotion and progression are three major steps of chemical carcinogenesis. During initiation stage DNA damage and cell proliferation happen. If DNA damage is not repaired or cells with unrepaired DNA damage are not removed by apoptosis, it may result in mutations. In promotion stage, initiated cells are proliferating which increases the risk of accumulation of mutations and development of malignant neoplasia (Figure 2). The third stage of chemical carcinogenesis is progression, this stage is distinguished by, genetic instability, rapid growth pattern, irreversibility, metastization, angiogenesis and morphological changes in cells (Oliveira et al., 2007).

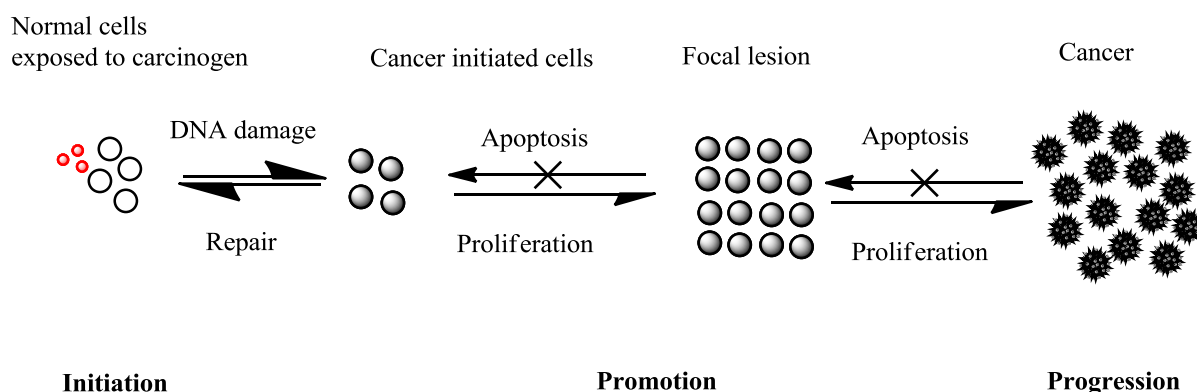


Figure 2. Chemical carcinogenesis (Oliveira et al., 2007)

2.2.1 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are the pernicious group to human health (Nollet, 2011). PAHs form a large group of organic compounds composed of two or more fused aromatic rings with only carbon and hydrogen atoms in their structure. Benzo(a)pyrene (BP, Figure 3) is a model compound of PAHs and it is used to study molecular effects of PAHs. The number of possible isomers increases with each additional ring, and yet further isomers are possible with the alkylation of PAHs (Haritash and Kaushik, 2009). An even greater variety of possible compounds are found in the wider group of polycyclic aromatic compounds (PACs) to which PAHs belong. PACs also include PAHs substituted with either one or several different functional groups

such as amino-, chloro-, cyano-, hydroxyl-, oxy- or thio- groups, as well as heterocyclic derivatives containing nitrogen, oxygen or sulfur in their aromatic structure (Vo-Dinh, 1989).

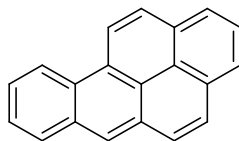


Figure 3. Benzo(a)Pyrene

2.2.2 Sources of Polycyclic Aromatic Hydrocarbons

PAHs are very stable compounds; these are produced due to incomplete combustion and pyrolysis of fossil fuel. PAHs are present in coal tar, crude oil, creosote and roofing tar. Anthropogenic combustion is also a big source for the emission of PAHs in the environment. PAH emission from domestic source is linked with burning of coal, oil and natural gas. Coal has less than one ratio of hydrogen-to-carbon and its incomplete combustion is a major source of PAHs production. Urban areas have more coal and wood burning which results into increased production of PAHs. Incomplete combustion of fuel of vehicles is also a significant source of PAH emission in air. The vehicles which run over diesel produce more PAHs than those of gasoline. Industries like waste incineration, cement manufacture and petrochemicals are important source of PAHs. Agricultural sources involve the burning of organic material under sub-optimal conditions which result into production of PAHs. Besides all these sources of PAHs, they are also produced by natural sources which include forest burning and volcanic eruption (Ravindra et al., 2008).

2.2.3 Exposure to PAHs and Their Toxicity Pathways

PAHs are present all around in environment. The main exposure routes of PAHs are via inhalation, food and by occupational means (IARC, 2010). Tobacco smoke is one of the main sources of PAHs because it has large amounts of PAHs (IARC, 2004). Direct heating of meat products also generate PAHs and quantity of PAHs depends on time and temperature of heating (IARC, 2010). Other food items may be contaminated by PAHs depending on their exposure to environment contaminated with PAHs. Industries in which workers are more prone to expose to PAHs include tar distillation, electrode manufacturing, aluminum production and wood impregnation (IARC, 2010).

PAHs have very less acute toxic effects; however, prolonged exposure to PAHs can cause development of cancer and immunosuppression. (IARC, 2010). According to Xue and

Warshawsky (2005) three pathways are involved in metabolic activation of PAHs to carcinogenic products. In the first step of first pathway, oxidation of double bond of PAHs is done by CYP P450 enzymes producing arene oxide. In second step this arene oxide is hydrolyzed by epoxide hydrolase forming dihydrodiol. In third step, double bond present adjacent to diol is oxidized by CYP enzymes generating reactive diol-epoxide. Diol-epoxide can bind to DNA by forming covalent bond and cause DNA damage. In second pathway PAHs are converted into radical cations by P450 peroxidase. In third pathway reactive oxygen species (ROS) are formed by PAHs via the formation of quinones by dihydrodiol dehydrogenases. These ROS may also take part in carcinogenesis (Xue and Warshawsky, 2005).

Several PAHs such as BP, benzo(e)pyrene and many others are pro-carcinogenic. In order to become carcinogenic BP must be metabolized by the enzyme cytochromes P450 mono-oxygenase most often by CYP1A1 (Nebert et al., 2000), epoxide hydrolase and dihydrodiol dehydrogenase to ultimate metabolite BP-7,8-dihydrodiol-9,10-epoxide (Figure 4) which bind covalently to the DNA. The formation of BPDE-DNA adduct causes DNA damage (Xue and Warshawsky, 2005).

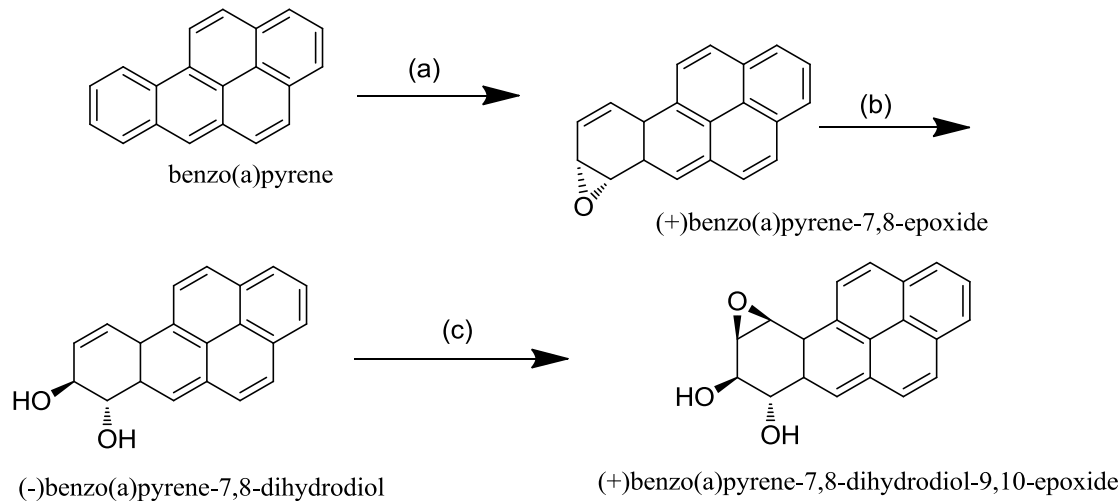


Figure 4. Metabolic activation of benzo(a)pyrene yielding benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide. Enzymes involved in reactions: a) and c) CYP1A1/CYP1B1; and b) Epoxide hydrolase

2.3 TP53 Gene and p53 Protein

TP53 gene resides on the chromosome 17 p¹³. It possesses 11 exons spanning 20 kilobases and encodes a nuclear phospho-protein of 53 kilo Dalton (kD), i.e. p53 protein. This gene belongs to a highly conserved gene family which also contains two other genes TP63 and TP73. However, p53 protein appears to be different from its family mates, since p53 protein deficient mice have the higher chances of developing the tumor while p63 and p73 deficient mice does not exhibit any tendency of the elevated tumor risk (Guimaraes and Hainaut, 2002).

2.3.1 p53 protein

Classical architecture of p53 protein possesses a sequence-specific transcription factor with an acidic N-terminus containing transactivation domain, a basic C-terminus oligomerization and regulatory domains and a hydrophobic central core which bind to specific DNA sequences (RRRCA/TA/TGYYY) (Guimaraes and Hainaut, 2002). The N-terminus domain of p53 protein is involved in the regulation of p53 protein by its binding to the MDM2 protein (Figure 5). The C-terminus domain of p53 protein contributes to the regulation of DNA binding activity. Both of these domains also contain the sites for post-translational modifications. N-terminal domains are involved in phosphorylations of p53 protein while C-terminal domains are involved in acetylation and also phosphoryations of p53 protein (Guimaraes and Hainaut, 2002)

Irrespective of the origin of the tumor, p53 seems to be the focal point among all genetic pathways involved in the cancer progression. Several p53 alterations are the point mutation, loss of allele and inactivation of protein by sequestration, viral antigen or cellular proteins. The complete data base of all mutations is present and maintained at the International Agency for Research on Cancer (IARC TP53 Database; www-p53.iarc.fr)(Petitjean et al., 2007).

Perhaps all details of mechanism by which the p53 is induced is not clearly understood, however it is accepted that those signals which causes DNA damage induces p53. Several studies show that by introduction of restriction enzymes in cells having wt TP53 gene results in accumulation of p53 protein. Genotoxic chemicals are known to cause p53 induction and activation. These include BP and especially its reactive metabolite, BPDE, which causes DNA damage by forming DNA adducts. Formation of BPDE is dose and time

dependent and also causes accumulation of the p53 protein the cells (Hainaut and Vahakangas, 1997). Several DNA-damage recognition molecules such as poly ADP ribose polymerase (PARP), the double stranded DNA dependent protein kinase (ds-DNAPK), and ataxia telangiectasia mutated (ATM) are involved in the activation of the p53 protein. When DNA is damaged PARP form the polymers of ADP-Ribose by using a co-substrate like nicotinamide adenine dinucleotide (NAD), this will indicate that it acts as an intermediate in the activation of the p53 protein (Hainaut and Vahakangas, 1997).

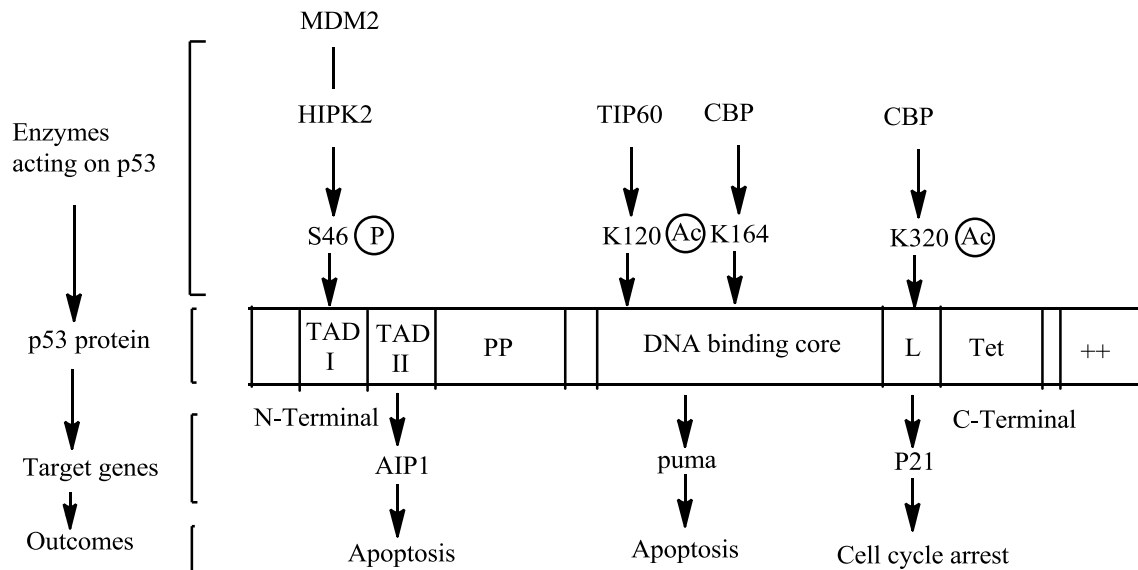


Figure 5. Effect of p53 modifying enzymes on p53 domains and their outcomes. TAD is transcription activation domain, PP stands for proline domain, L is the linker region, Tet is tetramerization domain and next to Tet is lysine rich domain. Ac and P means acetylation and phosphorylation respectively (Vousden and Prives, 2009).

2.3.2 Mutated p53 Protein

According to Olivier et al (2010) nearly half of human cancers have mutation in TP53 gene. It has been analyzed that DNA binding domain i.e. 102-292 amino acids of p53 protein is 97% mutated in all these cancers (Olivier et al., 2010). Tumor suppressor functions of p53 protein is altered because of mutation of DNA binding domain. Mutant p53 is accumulated leading to increase in proliferation of the cells (Bode and Dong, 2004) Petitjean et al. (2007) explored the mutation pattern on TP53 gene. Hot-spot mutations of TP53 gene are coupled with various carcinogens (Petitjean et al., 2007), for example, Ultra Violet (UV) light persuades a transformation from CC to TT in TP53 gene and Aflatoxin B1 (AFB1) prompts a conversion from G to T in 249 codon in hepatocellular carcinoma provoked by AFB1 (Vahakangas, 2003). TP53 gene differs from the other tumor suppressor genes in sense of mutation. Sometimes, it is missense, frame shift, non-sense mutations and deletion in TP53

gene which cause the replacement of single amino acid with the other. (Guimaraes and Hainaut, 2002).

Mutations in TP53 gene are also induced by many carcinogens which result into colonial growth of cancerous cells. PAHs present in cigarette smoke cause guanine to thymine transition in TP53 gene in lung cancer cases. Aflatoxin induces guanine to thymine transition in hepatocellular carcinoma. UV light induces cytosine:cytosine to thymine:thymine mutation in skin cancer patients. Besides this, carcinogens like AFB1 can induce such mutations in TP53 that mutated p53 prevents apoptosis after tumor necrosis factor α treatment (Bode and Dong, 2004).

The DNA binding domain is made up of two beta sheets which are sandwiched with the help of the loops and helices and is stabilized with the help of the zinc (Meplan et al., 2000). All codons of the DNA binding realm does not carry equal possibility of the mutations instead there are 6 hotspots which are detectable in the most of the cancers like codon 175, 245, 248, 249, 273 and 282 (Guimaraes and Hainaut, 2002). One possible explanation why these hotspots are involved in the mutation is Targeted Mutagenesis. Cytosine present in the 5 out of 6 hotspot is methylated which is followed by the deamination. This is mostly caused by the tobacco smoke (Hainaut and Pfeifer, 2001).

2.4 Regulation of p53 Protein in Cellular Stress

Several mechanisms are involved in the induction of p53 in response to cellular stress. Cellular stress increases the amount in p53 protein mainly due to post-translational modifications of p53. N and C-Termini are the phosphorylation sites of p53. The stability of p53 is regulated by phosphorylation of MDM2 binding site in N-Terminus of p53. The phosphorylation interfere the ability of p53 to bind with MDM2. This region of p53 is phosphorylated by several stress response kinases like ATM, ATR, Chk1 and Chk2 (Ou et al., 2005). These kinases are activated in critical situations damaging the DNA, like exposure to ionizing radiations or chemicals (e.g. BP). They are also critical for activation of p53 function. By the action of these kinases p53 is phosphorylated at various serine residues like Ser-15 and Ser-37 (Ljungman, 2000). Once the p53 is phosphorylated the MDM2 cannot down-regulate the function of p53. p53 is also stabilized by phosphorylation of its MDM2 binding domain by Chk2 which in turn is activated by ATM. The N-Terminus of p53 protein in human or mice can be phosphorylated in vitro by DNA-PK, JNK, P38, CK1 and CAK (Ou et al., 2005). Phosphorylation of C-terminus of p53 protein appears to be involved in activation of p53 protein rather than its stability. Phosphorylation of serine at 392 and 315 by casein kinase-II activates DNA binding and transcriptional function of p53. Phosphorylation of serine at 392 and 315 by CK-II enhances nuclear localization while with CDK drive nuclear export. De-phosphorylation of serine at 315 by phosphatases causes the accumulation of stable nuclear p53 (Ou et al., 2005).

2.4.1 Activation Pathways of p53 Protein

The damage of DNA by various adducts of carcinogens triggers activation of various cascades, which in turn activates phosphatidylinositol-3 kinase. This kinase phosphorylates the p53 protein at the attachment site of MDM2 and dissociates MDM2 from p53 protein. In some cases MDM2 itself is the substrate of the kinases (Figure 5). This pathway activates p53 protein. (Guimaraes and Hainaut, 2002, Kojima et al., 2008).

P14^{arf} protein binds to MDM2 protein and stops its repressing capacity, which promotes p53 protein, which ultimately promotes p21 activation (Figure 6). In most of the neoplastic tumors the gene which encodes the DNA kinase is methylated which resulted in the deregulation of the several pathways as; Ras/Rb/E2F pathway or c-Myc Pathway may lead to the activation of the P14^{arf} protein (Agrawal et al., 2006, Guimaraes and Hainaut, 2002).

Jnk2-pathway is mediated by the non-genotoxic signals and is poorly understood. Some radio-protective drugs activate p53 protein without interrupting the MDM2 protein pathway and it involves the Jun-N-terminal Kinase 2. This enzyme binds to p53 protein in an inactive form and act as ubiquitin ligase similar to MDM2 protein and initiate degradation of p53 protein (Figure 6). Activated Jnk2-kinase activates p53 phosphorylation and stabilization. (Oleinik et al., 2007). The activation of this pathway involves non-DNA damaging signals (Guimaraes and Hainaut, 2002).

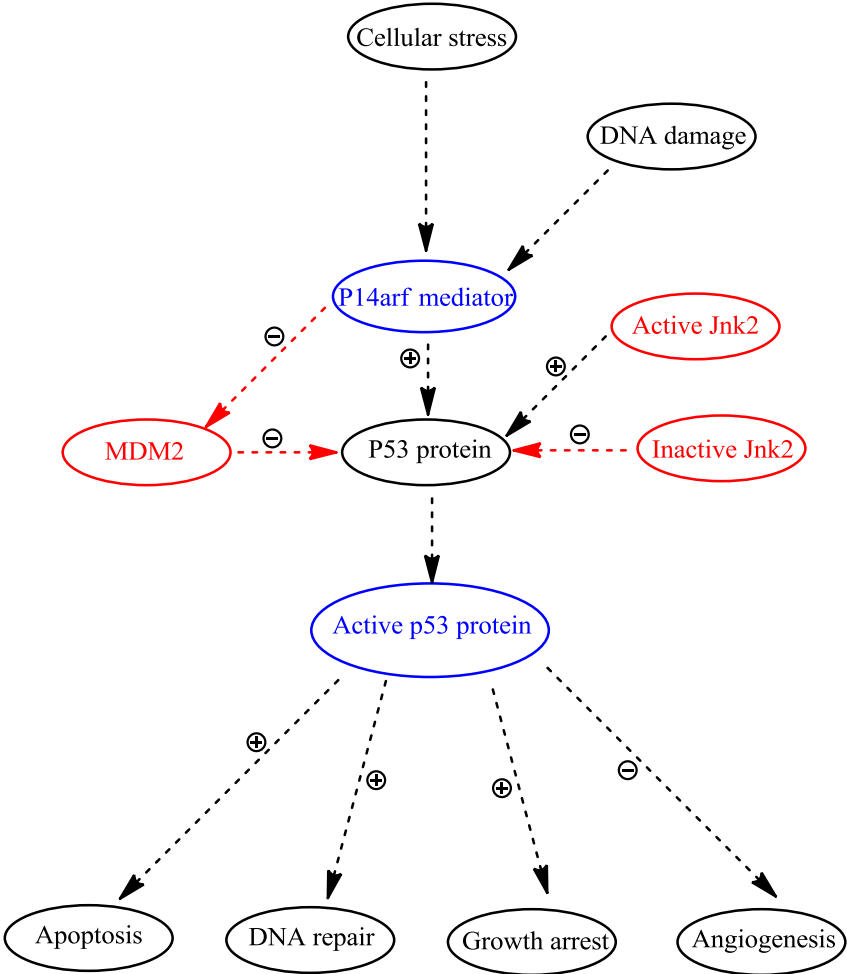


Figure 6. p53 activation pathways: for further details please see text (Guimaraes and Hainaut, 2002)

2.4.2 How MDM2 Protein Control p53 Protein?

The inhibitory effects of p53 on cell growth are monitored tightly. Different mechanisms exist to regulate its activity. However the major mechanism that regulates p53 activity is expressed at protein level and includes control of subcellular localization of p53 protein, post-translational modifications and conformational changes. The major component of negative p53 regulation is p53 interacting protein MDM2 (Michael and Oren, 2003). MDM2 regulates negatively the activity of p53; the importance of MDM2 can be illustrated by deleting MDM2 gene in mice. The deletion of MDM2 gene ensues in the embryonic lethality because of apoptosis in the embryo. This lethality can be counter reverse by p53 deletion. This demonstrates the importance of MDM2 in preventing the activation of p53 during development (Woods and Vousden, 2001).

The MDM2 is an oncoprotein, it inhibits the action of p53 protein. MDM2 degrade the p53 protein by means of ubiquitylation and proteasome-dependent pathway (Marine and Lozano, 2010). MDM2 binds to the N-terminal part of the p53 protein and ubiquitinates the C-terminal lysines. Ubiquitination leads to the export of p53 from nucleus to cytoplasm where it is degraded (Hollstein and Hainaut, 2010). p53 is expressed at very low level in the normal tissues due to its short half-life and is controlled by continuous removal of newly synthesized p53 protein through ubiquitin-dependent degradation by the proteasome. Ubiquitination involves cascades of many enzymes which are substrate specific known as ubiquitin protein ligases (E3s). Ring finger protein is the recently described group of E3s and acts as bridge to transfer ubiquitin from E2 ubiquitin conjugating enzyme to the substrate. MDM2 is a ring finger protein and functions as ubiquitin ligase enzyme for both p53 and itself also (Metzger et al., 2012).

Mutation in the ring finger part of the MDM2 protein causes stabilization of p53 and MDM2 itself by inhibiting their ubiquitination. The degradation of p53 by MDM2 is regulated by another protein P300 which facilitates the interaction of p53 and MDM2 in the cells (Woods and Vousden, 2001). Skp2 is another oncogenic protein which antagonizes the interaction between p300 and p53 by forming complex with p53 protein. So Skp2 negatively regulates the p300-mediated acetylation of p53 protein (Kitagawa et al., 2008). Cellular stress is the main factor which controls the role of p53 protein. Several studies indicate that negative regulation of DNA binding activity of p53 protein depends upon its C-Terminus, while the N-terminus has the role to regulate dissociation from DNA (Woods and Vousden, 2001).

2.4.3 p53 dependent apoptosis

Apoptosis is one of the significant responses after activation of p53 protein. Two different types of apoptotic pathways exist. These are intrinsic mitochondrial and extrinsic death receptor pathways. During intrinsic mitochondrial pathway, mitochondria receives death stimulus directly or indirectly via activation of pro-apoptotic proteins of Bcl-2 family like Bax and Bak. It will lead to the release of apoptotic proteins from mitochondria which activate caspase pathways which then result in apoptosis. However, in second pathway cell receives death signals via death receptors present on the cell membrane, these receptors then activate inhibitor caspases-8 which then leads to apoptosis. Apoptotic cell death under the activation of p53 follows usually intrinsic mitochondrial pathway (Figure 7). Once p53 protein is activated, it regulates the activation of proteins like Bax, Noxa and Puma (Amaral et al., 2010). Puma and Noxa belong to Bcl2 family and have role in induction of apoptosis. Under normal conditions, Puma participates in permeabilization of outer membrane of mitochondria, which result in calcium release from endoplasmic reticulum (Shibue et al., 2006). In one of the study conducted by Jeffers et al (2003), it was reported that Puma knockout mice showed deteriorated apoptosis induced by p53 protein (Jeffers et al., 2003). p53 protein has also the capability to induce apoptosis by suppressing the expression of anti-apoptotic genes like survivin which then also leads to activation of caspases pathway (Amaral et al., 2010)

The BP and several other PAHs such as cyclopenta[c,d]pyrene induce apoptosis in vitro as shown in Hepa1c1c7 liver cells (Solhaug et al., 2004b). The apoptosis is induced by the genotoxic BP in three steps. During the 1st step it is activated by the cellular enzymes to a reactive metabolite that covalently bind to the macromolecules. The 2nd step correspond to the damage and in the 3rd step cellular events occur which start from the primary cellular damage to the ultimate cell death (Solhaug et al., 2004a). Breast cancer cell lines which have unrepaired DNA damage undergo apoptosis or cell cycle arrest under the control of p53 protein. Caspases are the key enzymes which follow the cascade of complex reactions involved in the apoptosis. The effector caspases include caspase-3 and caspase-7 of which caspase-3 is thought to be essential for DNA fragmentation and for morphological changes in the cells during apoptosis (Boatright and Salvesen, 2003, Mc Gee et al., 2002).

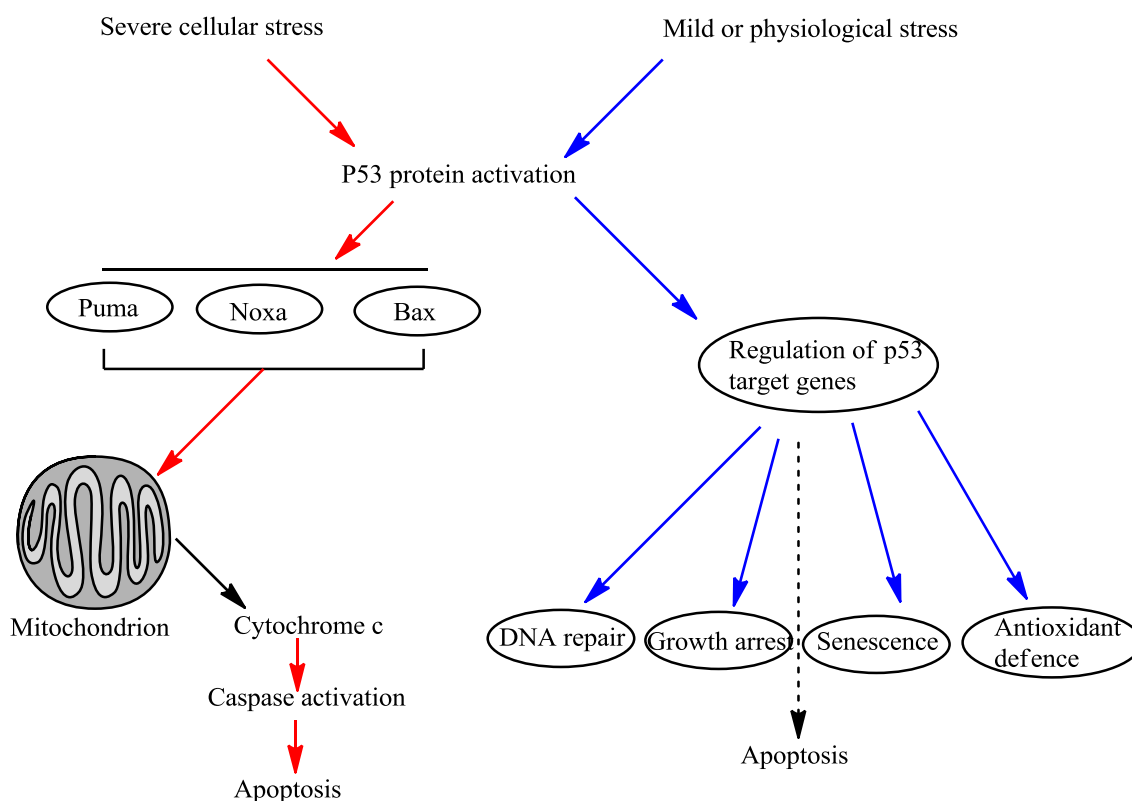


Figure 7. p53 dependent apoptotic pathway. The activation of p53 is related to the various cellular stresses; If it is severe then it persuades irreversible responses like apoptosis, with mild stress it prompts temporary stoppage of growth combined with effort to repair the cause of damage (Levine and Oren, 2009, Amaral et al., 2010).

There is much less studies available for the effects of the BP on the human breast cancer cells but its role in the apoptosis in rodent cells is studied (Ko et al., 2004). Breast cancer cell line MCF-7 which contains wild type p53 is capable of activating BP to BPDE and forming BPDE-DNA adducts (Ramet et al., 1995, Tampio et al., 2008). Further it is mentioned that p53 phosphorylation at Ser-392 is due to BP induced DNA damage (Melendez-Colon et al., 1999).

2.4.4 Post-Translational Modifications of p53 Protein and their Cellular Effects

p53 protein present in nucleus has very complex functions in cells. When cell receives stress stimuli in the form of oncogenic signals or DNA damage, the p53 protein suppresses the formation of tumor by apoptosis or by stopping cell cycle. It helps in prevention of proliferation of damaged cells. During stabilization and activation of p53, several post-translational modification occurs affecting e.g. the expression of target genes of p53protein (Bode and Dong, 2004).

Once p53 protein is activated, its phosphorylation and acetylation makes it more stable and it starts to accumulate in the nucleus. The most common form of p53 protein post-translational modifications include phosphorylations of serine and/or threonine residues, acetylation, ubiquitylation and sumoylation of lysine residue. During phosphorylation p53 protein is regulated by many proteins in a reversible manner. Protein kinases and phosphatases are the enzymes which are responsible for phosphorylation and dephosphorylation of p53, respectively (Bode and Dong, 2004).

Acetylation is the other form of post-translational modification of p53 protein which helps in stabilization of p53 protein. Acetylation of p53 protein occurs at its carboxy terminal after DNA damage. The carboxy terminal is also ubiquitylated by means of MDM2 protein. Deacetylation of p53 protein promotes ubiquitylation by MDM2 which results into its degradation. Transactivation of p53 protein is also thought to be suppressed by MDM2 protein by means of neddylation (Bode and Dong, 2004).

DNA binding domain of p53 protein has many sites for post-translational phosphorylation (Figure 5). Various phosphorylation/de-phosphorylation sites have been identified on DNA binding domain of p53 protein caused by ultraviolet light exposure. In human beings these sites comprise of Ser-149, Thr-150 and Thr-155. Transactivation domain comprise of Ser-6, Ser-9, Ser-15, Ser-20, Ser-33, Ser-37, Ser-46 Thr-18 and Thr-81 phosphorylation sites at amino terminal. Phosphorylation sites at carboxy terminal domain comprise of Ser-315 and Ser-392. Cellular responses are determined on the basis of phosphorylation pattern produced by specific stimuli. Casein kinase-1 causes the phosphorylation of Ser-6, Ser-9 and Thr-18. C-Jun amino terminal kinases induce phosphorylation at Thr81. De-phosphorylation of p53 protein at Ser-376 and Thr-55 by ionizing radiations is also considered to be involved in activation of p53 protein (Bode and Dong, 2004). Serine/threonine protein phosphatase-1 is the enzyme which dephosphorylates p53 protein at Ser-15 and Ser-37 by immunoprecipitation. Dephosphorylations of p53 protein result in increased cell survival and decreased apoptosis (Li et al., 2006).

Phosphorylation of p53 protein is a transient event. p53 protein is also phosphorylated in normal human cells at Ser-9, Ser-15, Ser-20 and Ser-372 during G1 phase of cell cycle, in G2 phase Ser-37 and Ser-392 is phosphorylated and during S-phase only Ser-392 is phosphorylated. While acetylation of p53 protein is occurring at G0 phase only. On the other hand, cancerous cells of human origin show hyperphosphorylation and hyperacetylation. One

of the previous studies on MCF-7 breast cancer cell line with wild type p53 protein have shown that metabolite of BP i.e. BPDE activates the p53 protein by its phosphorylation (Tampio et al., 2008).

3 AIM OF THE WORK

Breast cancer is the most haunting cancer in women across the world. There is evidence which shows that PAHs present in tobacco smoke is one risk factor for this disease. Our aim in this study was to uncover the BP-induced mechanisms of carcinogenesis and cellular protection against BP-induced carcinogenesis in breast tissue. BP has to be converted to BPDE, a mutagenic metabolite, to cause carcinogenic effects. It has been known that p53 protein is induced in the breast cells after the exposure to BP. p53 protein has been known to be linked with many important actions like apoptosis after its induction / post-translational modifications (phosphorylation, acetylation etc.). This study aimed to unhide p53-related cellular responses i.e. post-translational modifications (phosphorylation at Ser-15 and Ser-392) of p53 protein *in-vitro* in human breast cancer cell lines (MDA-MB-231, MDA-MB-468, ZR-75-1 and T-47-D) after exposure to BP. In addition, our intention was also to find out whether there are differences in p53 response after BP exposure in the studied cell lines.

4 MATERIALS AND METHODS

4.1 Breast Cancer Cell Lines

MDA-MB-231 and MDA-MB-468 cell lines were isolated from adenocarcinoma of 51 year old woman by plural effusion in mid-1970. ZR-75-1 and T47-D cell lines were isolated from ductal carcinoma of 63 and 54 years old women, respectively, from metastatic ascites in late-1970 (Cailleau et al., 1978, Cailleau et al., 1974, Engel and Young, 1978, Keydar et al., 1979). (See Table 1)

Table 1. Features of human breast cancer cell lines

Cell lines	Primordial cancer	First described
MDA-MB-231	Adenocarcinoma	(Cailleau et al., 1974)
MDA-MB-468	Adenocarcinoma	(Cailleau et al., 1978)
ZR-75-1	Ductal carcinoma	(Engel and Young, 1978)
T-47-D	Ductal carcinoma	(Keydar et al., 1979)

4.2 Growth Media

Two types of media were used for cell culturing namely Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640. Medium details for each cell line are given in Table 2.

Table 2. Details of cell culturing media

Ingredients	Cell lines			
	MDA-MB-231	MDA-MB-468	ZR-75-1	T-47-D
Medium	DMEM	DMEM	RPMI1640	RPMI1640
FBS	9%	9%	9%	9%
Gentamycin	10µg/ml	10µg/ml	10µg/ml	10µg/ml
L-glutamine	0.6g/L	0.6g/L	0.6g/L	0.6g/L
Glucose	4.5g/L	4.5g/L	2g/L	2g/L
Insulin	-	-	-	8 µg/ml
HEPES	-	-	10mM	10mM
Na-pyruvate	-	-	1mM	1mM
Lot. N°	9MB102	9MB102	8MB204	8MB204

4.3 Particulars of Solutions Utilized

4.3.1 Benzo(a)pyrene Solutions

Stock solution of 10 mM solution of BP was diluted to 5 mM and 0.5 mM solutions. For making 5 mM BP solution 75 μ l of 10 mM BP was added in 75 μ l of Dimethyl Sulfoxide (DMSO). For producing 0.5 mM BP solution 20 μ l of 5 mM BP was added in 180 μ l of DMSO.

4.3.2 EMSA B Buffer Solution / Lysis Buffer

20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.6; 20% glycerol; 500 mM NaCl; 1.5 mM MgCl₂; 0.2 mM Ethylene Diamine Tetra Acetic acid (EDTA) pH 7.6; 0.1% NP 40. Lysis buffer was supplemented with protease inhibitors according to manufacturer's instructions (Roche Complete Protease Inhibitor Cocktail Tablets).

4.3.3 Bovine Serum Albumin (BSA) For Protein Assay

BioRad protein dye (Coomassie Brilliant blue) was diluted 1:5 (one part dye concentrate + 4 parts H₂O) before used in the measurement of protein concentration. In addition, the diluted dye was filtered. BSA (10mg/ml) stock solution was already available in laboratory. Protein standards were prepared from stock solution according to table 3.

Table 3. Preparation of BSA standards

Ready Standards (mg/ml)	BSA (μ l)	Dilution (xmg/ml)	Milli-Q water (μ l)	
STD-6	0.5	50	10	950
STD-5	0.4	400	0.5	100
STD-4	0.3	375	0.4	125
STD-3	0.2	250	0.3	125
STD-2	0.1	250	0.2	250
STD-1	0.05	250	0.1	250

4.3.4 Western Blot Liquids and Corresponding Materials

- *Tris-Hydrochloric Acid (Tris-HCl) 1.5 M, pH 8.8:* 36.3 g Tris base was dissolved in water, pH was adjusted first with 37% HCl and finally to 8.8 with 1M HCl. Thereafter final volume was made to 200 ml with water

- **Tris-HCl 0.5 M, pH 6.8:** 12.1 g Tris base was dissolved in water, pH was adjusted finally to 6.8 with 1M HCl. Final volume was made to 200 ml with water
- **Sodium Dodecyl Sulfate (SDS) Reducing Sample Buffer (4XSB) / Laemmli Buffer:** 5 ml of 0.5 M Tris HCl pH 6.8; 4 ml of glycerol; 0.8 g of SDS; 40 mg of bromophenol blue and 800 µl of β-mercaptoethanol.
- **1X Running Buffer:** 25 mM Tris base; 192 mM glycine and 0.1% SDS, pH was first adjusted with 37% HCl and finally to 8.3 with 1 M HCl
- **1X Transfer Buffer:** 25 mM Tris base; 192 mM glycine and 20% ethanol.
- **Tris Buffered Saline (TBS) 10X (Concentrated TBS):** 60.6 g Tris base and 116.8 g NaCl was dissolved in H₂O and pH were adjusted with 37% HCl to 7.4. Final volume was made with H₂O to 1000 ml.
- **10% Tween Stock:** 10 g of Tween-20 was dissolved in 100 ml of water
- **Tris Buffered Saline Tween (TBST) 0.1% V/V:** 10 ml of 10% Tween stock was added to 1000 ml of 1 x TBS.
- **5% Milk TBS 0.1% Tween:** 25 g of non-fat milk powder was dissolved in 1 x TBS and 5 ml of 10% Tween was added. Final volume to 500 ml was adjusted with 1 x TBS.
- **0.5% Milk TBS 0.1% Tween:** 1 ml of 5% milk TBS Tween +9 ml of 0.1% Tween in 1 x TBS.
- **Enhanced Chemiluminescence (ECL) Western Blotting Detection Reagents:**
ECL+ (GE Healthcare): solution-A and solution-B
- **Horseradish peroxidase conjugated secondary antibodies:** See Table 4
- **1X Blocking Buffer:** 5% milk in TBST
- **Primary Antibodies:** See table 4
- **Polyclonal / Monoclonal Antibody Buffer 0.5% milk in TBST**
- **Polyvinylidene Fluoride (PVDF) membrane**
- **VWR Blotting Paper grade 703**

4.3.5 Gels Utilized In Immunoblotting

- **Lower Gel (12%):** 3.35 ml water; 2.5 ml of 1.5 M Tris-HCl having pH 8.8; 100 µl of 10% Sodium Dodecyl Sulfate (SDS); 4 ml of acrylamide / bis-acrylamide

30%; 50 µl of 10% Ammonium Persulfate (APS) and 5 µl of N, N, N', N'-tetramethylethylenediamine (TEMED).

- **Upper Gel:** 3.05 ml water; 1.25 ml of 0.5 M Tris-HCl having pH 6.8; 50 µl of 10% SDS; 650 µl of acrylamide / bis-acrylamide 30%; 25 µl of 10% APS and 7.5 µl of TEMED.

4.3.6 Antibodies Utilized In Immunoblotting

Details of utilized primary and secondary antibodies are shown in Table 4.

Table 4. Details of primary and secondary antibodies utilized

Primary antibody	Dilution	Company	Secondary antibody*	Dilution	Company
Anti-p53-DO-7	1:2000	NovoCastra laboratories	Anti-mouse IgG	1:2000	Amersham BioSciences
Anti-phospho-Ser15-p53	1:2000	Cell Signaling	Anti-mouse IgG	1:2000	Amersham BioSciences
Anti-phospho-Ser392-p53	1:2000	Cell Signaling	Anti-rabbit IgG	1:2000	Cell Signaling or Calbiochem
Anti-β-actin	1:2 million	Sigma	Anti-mouse IgG	1:2000	Amersham BioSciences

*Secondary antibodies were conjugated with horseradish peroxidase

4.4 Cell Culture and Exposure to BP

MDA-MB-231 and MDA-MB-468 cells were cultured in DMEM containing 4.5 g/l glucose and 0.6 g/l L-glutamine (BioWhittaker, Belgium) and supplemented with 10µg/ml gentamicin (Gibco, UK) and 9% heat inactivated foetal bovine serum (FBS, Gibco, UK) at 37°C in a cell culture incubator (Revco Scientific, NC, USA) with a humidified atmosphere holding 95% air and 5% CO₂. ZR-75-1 cell line was cultured in RPMI 1640 containing 2g/l glucose (BioWhittaker, Belgium), 0.3g/L L-glutamine (BioWhittaker, Belgium), before cell culture it was supplemented with 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (BioWittaker, Belgium), 1mM Na-pyruvate (BioWittaker, Belgium), 10µg/ml gentamicin (Gibco, UK) and 9% heat inactivated FBS (Gibco, UK) at 37°C in a cell culture incubator (Revco Scientific, NC, USA) with a humidified atmosphere holding 95% air and 5% CO₂.

T-47-D cell line was cultured in same media as used for ZR-75-1 in addition holding 8µg/ml insulin (Gibco, UK). For sub-culturing cells were detached with 0.05% trypsin-0.02%EDTA. The sub-culturing was performed on 6-well plates. Cells were cultured 24 h before the exposure in a cell culture incubator. During experiments cells were exposed to 1µM and 10µM BP (Sigma) dissolved in DMSO (Sigma) in a duplicate fashion for 48h. Control cells were exposed to 0.2% DMSO, the solvent of BP (Table 5).

Table 5. Scheme how cells were exposed to BP (1 and 10 µM) for 48 h on 6-well plates.

(I) <u>Control</u> (0.2% DMSO) 2.5 ml medium 5 µl DMSO	(I) <u>1 µM BP</u> 2.5 ml medium 5 µl 0.5 mM BP	(I) <u>10 µM BP</u> 2.5 ml medium 5 µl 5 mM BP
(II) <u>Control</u> (0.2% DMSO) 2.5 ml medium 5 µl DMSO	(II) <u>1 µM BP</u> 2.5 ml medium 5 µl 0.5 mM BP	(II) <u>10 µM BP</u> 2.5 ml medium 5 µl 5 mM BP

4.5 Total Cell Protein Fractions from BP Exposed Cultures

Following exposure to BP, the cells were washed with ice cold PBS and then adherent cells were detached by scrapping in 35 µl EMSA B buffer complemented with protease inhibitors (Complete Mini Protease Inhibitor cocktail tablets, Roche Diagnostics GmbH, Mannheim, Germany). Thereafter, samples were incubated for 30 min on ice. EMSA B buffer caused the lysis of cells and release of proteins. Protease inhibitors slow down proteolysis. Whole process was carried out by placing the samples on ice or at 4°C. Cell lysate was obtained by centrifugation with micro-centrifuge at 13000 rpm for 15 min at 4°C. Eppendorf tubes containing samples were removed gently from centrifuge and placed on ice. Supernatant was pipetted in new eppendorf tube placed already on ice. The pellet was discarded. Supernatants containing protein were stored at -80°C until analysed by immunoblotting.

4.6 Protein Measurement by Standard Microtiter Bradford Analysis

Bradford protein analysis is a well-known method for determination of protein concentration. The basic principle in the technique involved was use of Coomassie Brilliant Blue G-250 dye which bound to protein that ultimately resulted into shift in the absorption maximum of dye from 465 nm to 595 nm and this increase in the absorption at 595 nm was

measured (Bradford, 1976). With increased concentration of protein in samples the absorbance of light at 595 nm increased linearly.

Protein concentration was measured in collected supernatant using BSA as standard protein (Table 3). Five BSA standards with known protein concentration were prepared in Milli-Q water from BSA stock solution (10mg / ml). Protein samples were diluted ten times with Milli-Q water and placed constantly on ice at 4°C. MDA-MB468 samples were diluted five times since cytotoxicity by BP. Coomassie Brilliant Blue G-250 dye reagent was prepared by diluting it five times with Milli-Q water and filtered through whatman#1 filter paper. 10 µl of each standard, sample and blank (water) were pipette as duplicate on microtiter 96-well plate. 200 µl of Coomassie Brilliant Blue G-250 diluted dye solution was added with multi-channel pipette to each well containing sample, blank and standard protein solutions. Plunger was depressed repeatedly to mix reagents well and then tips of pipette were replaced with clean tips for next set of wells. Microtiter plate containing reagents were incubated at room temperature for 5 min, before measuring absorbance.

The absorbance was measured at 595 nm with Spectrophotometer (ELx800UV-Visible Absorbance Microplate Reader). Prior measuring absorbance the instrument was allowed to warm up for at least 15 min. The absorbance of BSA standards was plotted as a function of its theoretical concentration. Linear regression was determined by following equation.

$$y = mx + b$$

Whereas y is absorbance and x is protein concentration in sample solutions. This equation was used to calculate concentration of protein in samples based on measured absorbance by taking into account the dilution factor. Until the start of further immunoblotting analysis samples were stored at -80°C.

4.7 Immunoblotting of p53, Phospho-Ser15-p53 and Phospho-Ser392-p53 proteins

Western blotting or Immunoblotting is a technique that has been employed for identification of specific proteins separated from one another by gel electrophoresis based on their molecular weight. After gel electrophoresis, proteins are transferred to membranes from which specific proteins are recognized by antibodies.

4.7.1 Gel Electrophoresis

One dimensional SDS-PAGE (Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis) method was used in this study. In SDS-PAGE proteins are separated according to their molecular weight. Polyacrylamide gels were formed by polymerization of two compounds acrylamide and bisacrylamide. Bisacrylamide was used as cross linking agent. The ratio used between acrylamide and bisacrylamide was 37.5:1 (30% acrylamide and 0.8% bisacrylamide). The polymerization process was initiated by addition of APS which produced free radicals in TEMED. Gels were polymerized between two glass plates in a gel caster. First lower gel was made by pouring the mixture in between glass plates settled in gel caster. Small quantity of water was also poured on top of lower gel to prevent it from drying.

It was left for 35 min until the gels solidified. With the help of filter paper distilled water above the gel was soaked and mixture of upper gel was poured and a comb inserted at the top, which created the wells for sample loading. After polymerization comb was removed. Extra care was taken for prevention of neurotoxicity from acrylamide during formation of gels. Gel combs were removed and wells were rinsed with distilled water. 1 X running buffer (diluted from 10X buffer with distilled water) was added in the chamber (cathode buffer). Gels were settled on a rack and then in the chamber with shorter glass surface pointing inward of chamber. Running buffer was also added inside the chamber (anode buffer). Gels were submerged in running buffer.

In order to make antibody accessible to epitope SDS-Reducing sample buffer (4XSB) / Laemmli Buffer was used. Samples were taken from -80°C and placed on Ice. Same amount of sample protein (10 or 20 µg) was pipette in new eppendorf tubes. For denaturation of protein the samples were heated with SDS-Reducing sample buffer (4XSB) / Laemmli Buffer in a heat block at 97°C for 4 min. Samples were centrifuged for 10 sec before and after the heating at centrifugations force of 13000 rpm.

Samples were loaded into wells using normal pipettes with special tips designed for this purpose, from left to right 1st well was reserved for loading molecular weight marker (4 µl) which was mixture of proteins with defined molecular weights. Extra care was taken to avoid poking of well bottom to avoid distortion of band. Lid was placed over the chamber. SDS present in migration buffer covered the protein molecules present in sample with a negative charge. Gel was run for 60 min at 200V. The applied electric current moved the

negatively charged protein molecules downward towards the anode and hence resulted into separation of protein molecules onto SDS-PAGE in the form of band.

4.7.2 Transfer (Blotting)

After gel electrophoresis, gel on glass plate were placed in cold transfer buffer solution for equilibration, meanwhile PVDF membranes were pre-treated by putting into absolute ethanol for 5 min, and then these membranes were placed to transfer buffer solution for 10 min. Semi-dry transfer process was used to transfer proteins form gel to PVDF membranes. The size of PVDF membrane and filter papers were cutted according to size of gel. Special precaution was taken to avoid direct contact of fingers with membrane since it would hinder transfer of protein form gel to membrane. During this process gel and membrane were sandwiched between wet filter papers (in transfer buffer) as follows (Figure 8);

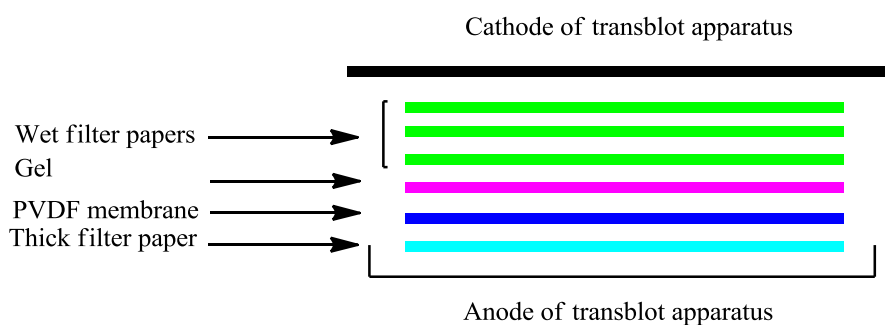


Figure 8. Western blotting arrangement

These were clamped tightly to ensure absence of air bubbles. More precisely air bubbles were removed by rolling the glass rod on sandwich of gel and PVDF membrane and wet filter papers. All these settings were made over anode of trans-blot apparatus (Trans-Blot® SD, Semi-Dry Transfer Cell, BioRad). Cathode was placed over it. The electrophoretic transfer was run at 20V for 25 min. The applied electric field caused movement of negatively charged proteins towards anode i.e. from gel onto a sturdy support, the PVDF membrane which blotted the proteins form gel.

4.7.3 Blocking of PVDF Membrane

Blocking was performed to avoid non-specific background binding of primary and secondary antibodies to PVDF membrane. After successful transfer of proteins to PVDF membrane, it was placed in distilled water for two minutes. Gel and filter papers were discarded. In this study, blocking was done in 5% non-fat cow milk–TBS–0.1% Tween (10

ml). PVDF membranes were incubated in blocking buffer for 75 min at room temperature in a horizontal mixer. Milk was discarded after blocking.

4.7.4 Incubation with Primary Antibody

Primary antibodies were used to locate protein of interest, in this study proteins of interest were p53, phospho-ser15-p53, phospho-ser-392-p53 and β -actin as a loading control. Antibodies were diluted (1:2000) in TBST containing 0.5% milk to optimize the results. Anti-p53-DO-7 (NovoCastra laboratories), anti-phospho-ser15-p53 (Cell Signaling) and anti-phospho-ser-392-p53 (Cell Signaling) were used for detection of p53, phospho-ser15-p53 and phospho-ser-392-p53 proteins on PVDF membrane respectively (Table 4). For detection of loading control β -actin, Anti- β -actin (1:2 million dilution, Sigma) was used as primary antibody. Membranes were incubated in primary antibody (10ml) solution at 4°C overnight in a horizontal mixer. The PVDF membranes were placed in TBST after treatment with primary antibody in horizontal mixer for ten minutes. Washing was repeated three times.

4.7.5 Incubation with Secondary Antibody

Secondary antibodies were used to detect primary antibodies bound to studied protein. Secondary antibodies were diluted (1:2000) in TBST to optimize the results. Anti-mouse IgG (Amersham BioSciences) was used to find anti-p53-DO-7 (NovoCastra laboratories), anti-phospho-ser15-p53 (Cell Signaling) and anti- β -actin (Sigma) antibodies. Anti-rabbit IgG (Cell Signaling or Calbiochem) was used for detection of anti-phospho-ser-392-p53 protein (Table 4). The secondary antibodies were conjugated with a reporter enzyme horseradish peroxidase. Membranes were incubated in secondary antibody at room temperature for 60 min in a horizontal mixer. The PVDF membranes were placed in TBST after treatment with secondary antibody in horizontal mixer for ten minutes. Washing was repeated three times.

4.7.6 Detection

For horseradish peroxidase conjugated antibodies ECL+ Plus kit (Amersham BioSciences) was used to detect protein bands. 1.95 ml of liquid-A and 50 μ l of liquid-B of ECL+ Plus kit were mixed in a small cup. This liquid was poured on PVDF membrane and was incubated at room temperature for five minutes. The liquid was removed and then PVDF membrane was placed on a glass plate. A transparent film was placed over PVDF membrane. The horseradish peroxidase conjugated with secondary antibodies catalyzed the

chemiluminescent substrate into a reagent which emitted light and hence proteins of interest could be detected. Protein bands of p53, phospho-ser15-p53, phospho-ser-392-P53 and β -actin were visualized (Amersham BioSciences) with ImageQuantTM RT ECLTM imaging system (GE Healthcare, USA).

4.7.7 Densitometry Analysis

Densitometry analysis was carried out using QuantityOne® program (1-D Analysis Software, version 4.6.3, Bio-Rad Laboratories Inc., USA). The digital images from ECL+ Plus system were loaded into QuantityOne® program. In every image of sample, the ratio between the densitometry values of the protein and the corresponding loading control (e.g. p53/b-actin) was analysed. The density was calculated in terms of intensity of image. The results are shown as the ratio of original densitometry values.

All experiments were repeated two times for all cell lines i.e. DMSO exposed, 1 μ M BP exposed and 10 μ M BP exposed.

5 RESULTS

5.1 Effects of BP Exposure on Cell Morphology

Cells were checked under microscope after the end of exposure and photos were taken (Figure 9-12). Figure 9-12 shows the photo shots of MDA-MB-231, MDA-MB-468, T-47-D and ZR-75-1 cells with (1 μ M and 10 μ M) and without BP exposure. With 1 μ M exposure of BP, there was no much change in morphology except in ZR-75-1 (cells were bursted) cell line. While with 10 μ M exposure MDA-MB-468 and ZR-75-1 cells bursted, on the other hand MDA-MB-231 and T-47-D cells seem quite normal in shape when compared to control cells.

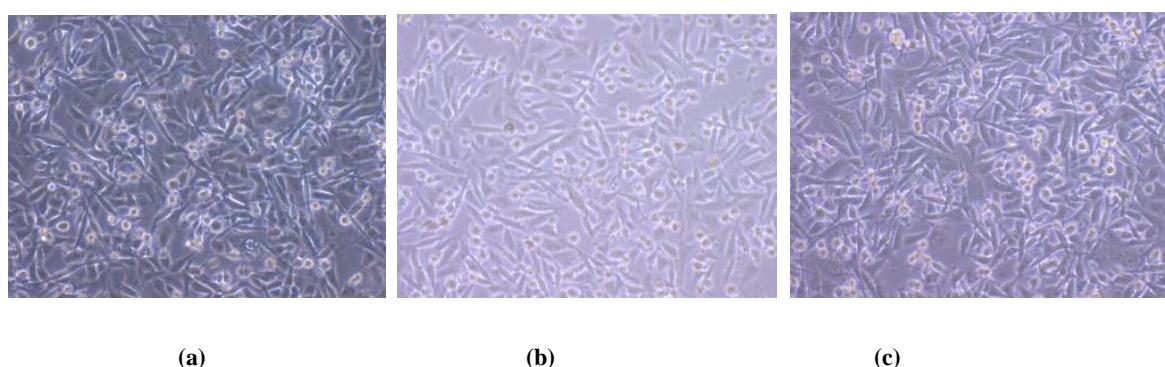


Figure 9. MDA-MB231 cell line. Cells after exposure to BP for 48 h: a) Control (DMSO), b) 1 μ M BP and c) 10 μ M BP.

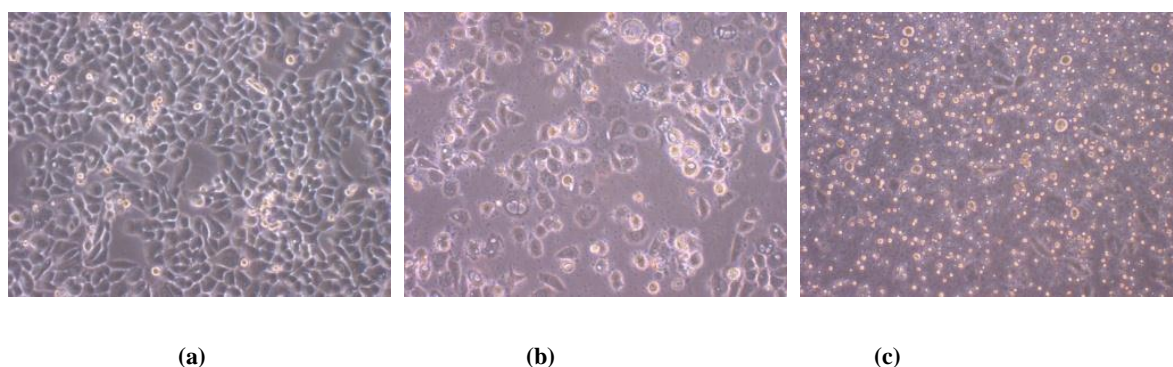


Figure 10. MDA-MB-468 cell line, Cells after exposure to BP for 48 h: a) Control (DMSO), b) 1 μ M BP and c) 10 μ M BP.

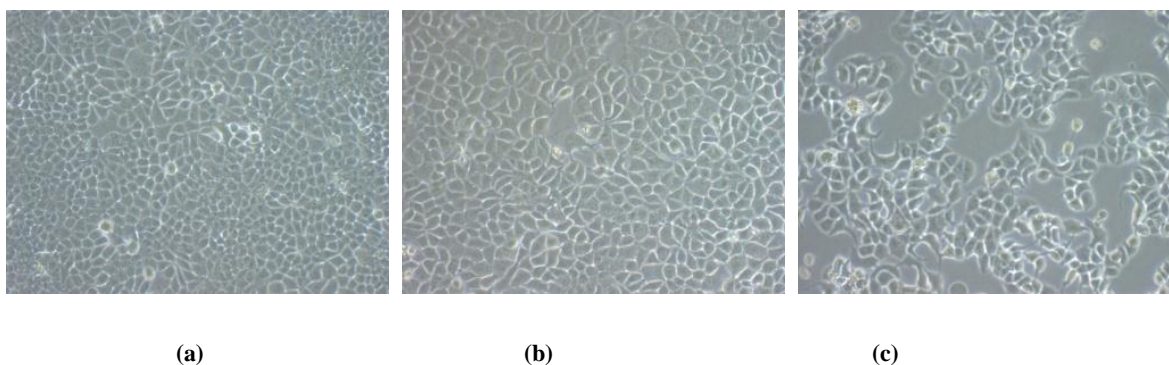


Figure 11. T-47-D cell line. Cells after exposure to BP for 48 h: a) Control (DMSO), b) 1 μ M BP and c) 10 μ M BP.

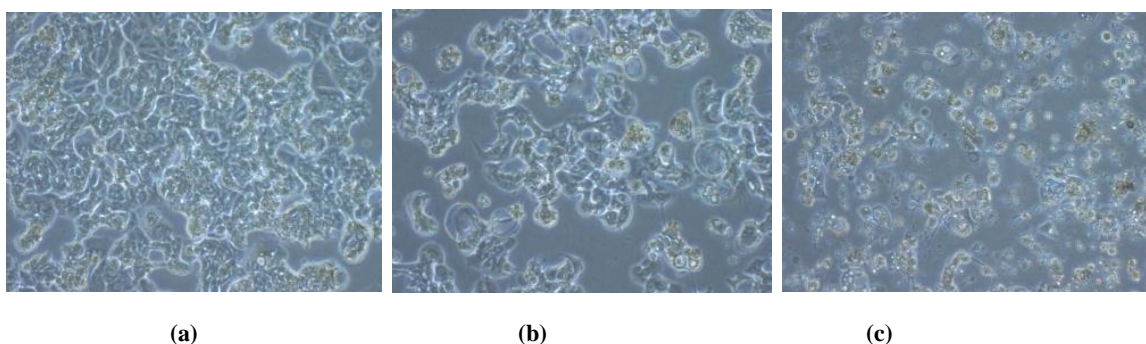


Figure 12. ZR-75-1 cell line. Cells after exposure to BP for 48 h: a) Control (DMSO), b) 1 μ M BP and c) 10 μ M BP

5.2 Effects of BP Exposure on p53 protein

In this study p53 was induced after exposure to BP (1 and 10 μ M) for 48h in all studied breast cancer cell lines, except in case of MDA-MB-231 cells which contained mutated TP53 gene, no clear induction was achieved. A notable increase was observed in ZR-75-1 cells at 10 μ M exposure to BP. An increase in the amount of p53 protein was also observed in both MDA-MB-468 (at both 1 μ M and 10 μ M exposure) and in T-47-D cells (more specifically at 1 μ M and less specifically at 10 μ M). In T-47-D cells BP-induced p53 induction was not as clear as in ZR-75-1 and MDA-MB-468 cells. For further details see Figure 13 and Table 6.

Table 6. Effect of BP on the induction of p53 protein and its phosphorylation in breast cancer cell at 48h

Outcomes	T-47-D	MDA-MB-468	ZR-75-1	MDA-MB-231
p53 protein	Not very clear	yes	yes	no
phospho-ser15-p53	yes	yes	least	yes
phospho-ser392-p53	yes	yes	yes	no

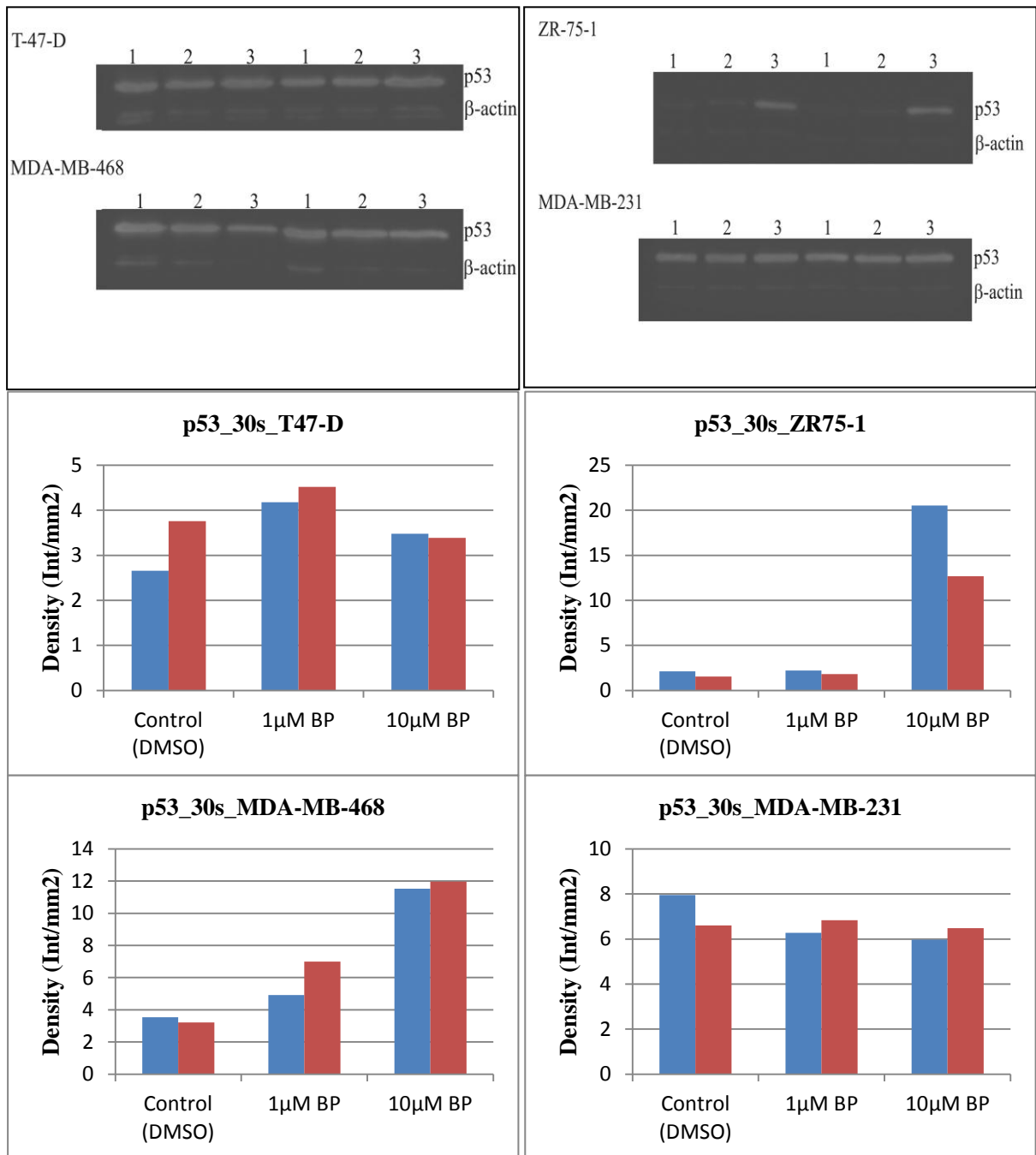


Figure 13. Immunoblotting of p53 protein gained from whole cell protein fractions (10-20 μg) of various breast cancer cells. Cells were exposed to 1 μM and 10 μM BP in duplicates for 48h. Numbers describe (1) control (2) 1 μM BP (3) 10 μM BP. Bar charts are drawn from densitometry values of immunoblotting results. Results are shown as the ratio of densitometry values of p53 protein and corresponding loading control. Duplicate results are shown as blue and red colors.

5.3 Effects of BP Exposure on Phosphorylation of p53 protein

The consequences of BP exposure on p53 phosphorylation at Serine -15 and Serine-392 in various breast cancer cell lines were also analyzed in this study. Anti-phospho-Ser15-p53 and anti-phospho-Ser392-p53 antibodies were utilized to detect corresponding protein samples of all cell lines. Phosphorylation of p53 at Ser15 was increased in all studied cell lines after BP exposure for 48 h. Lowest phosphorylation level of p53 at Ser15 was found in ZR-75-1 cells after BP exposure for 48h (Figure 14).

Phosphorylation of p53 at Ser392 was clear in MDA-MB-468 cells after exposure to BP for 48h. ZR-75-1 cells showed also increase in phosphorylation at Ser392 due to wt (wild type) TP53 after exposure to BP for 48h. Slight increase in phosphorylation at Ser392 was also found in T-47-D cells after exposure to BP for 48h, but it was not very clear. BP, after exposure for 48 h, did not increase phosphorylation of p53 at Ser392 in MDA-MB-231 cells (for further details, see Table 6 and Fig. 15).

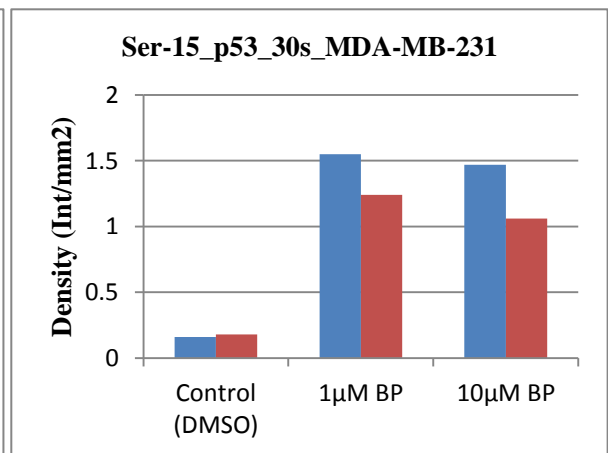
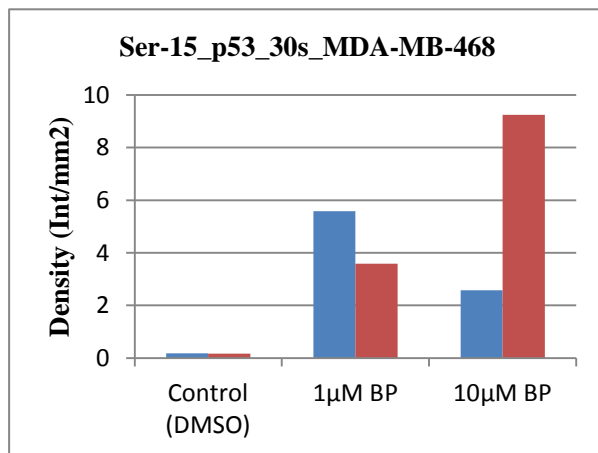
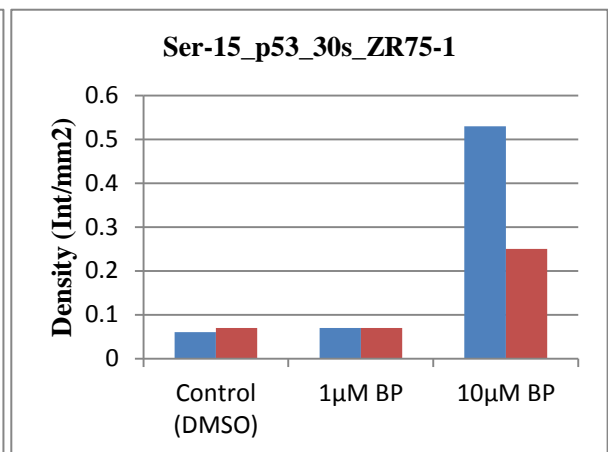
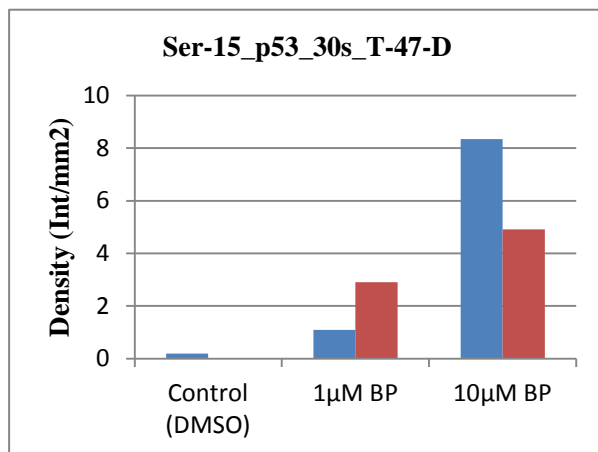
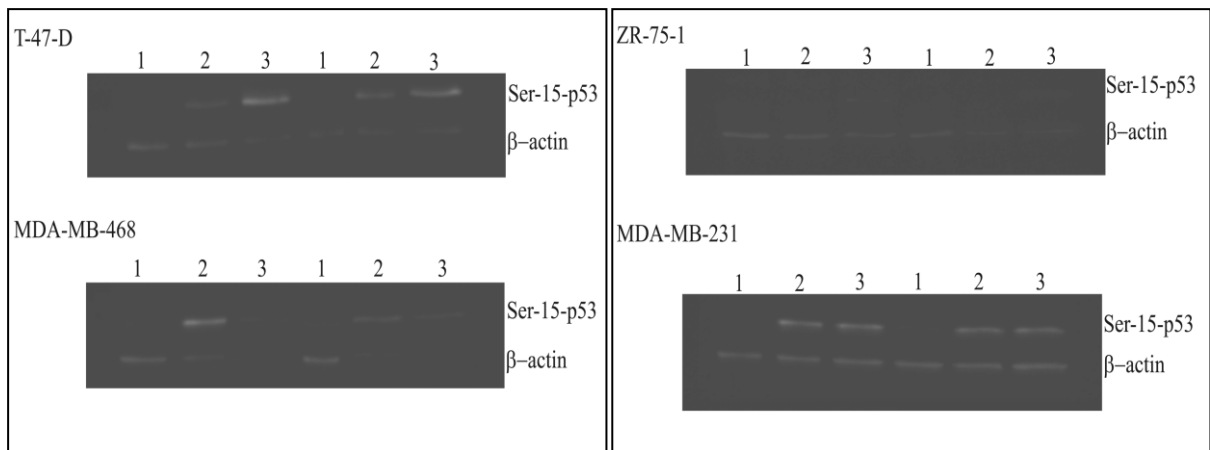


Figure 14. Immunoblotting of phosphoSer15-53 protein gained from whole cell protein fractions (10-20 μg) of various breast cancer cells. Cells were exposed to 1 μM and 10 μM BP in duplicates for 48h. Numbers describe (1) control (2) 1 μM BP (3) 10 μM BP. Bar charts are drawn from densitometry values of immunoblotting results. Results are shown as the ratio of densitometry values of p53 protein and corresponding loading control. Duplicate results are shown as blue and red colors.

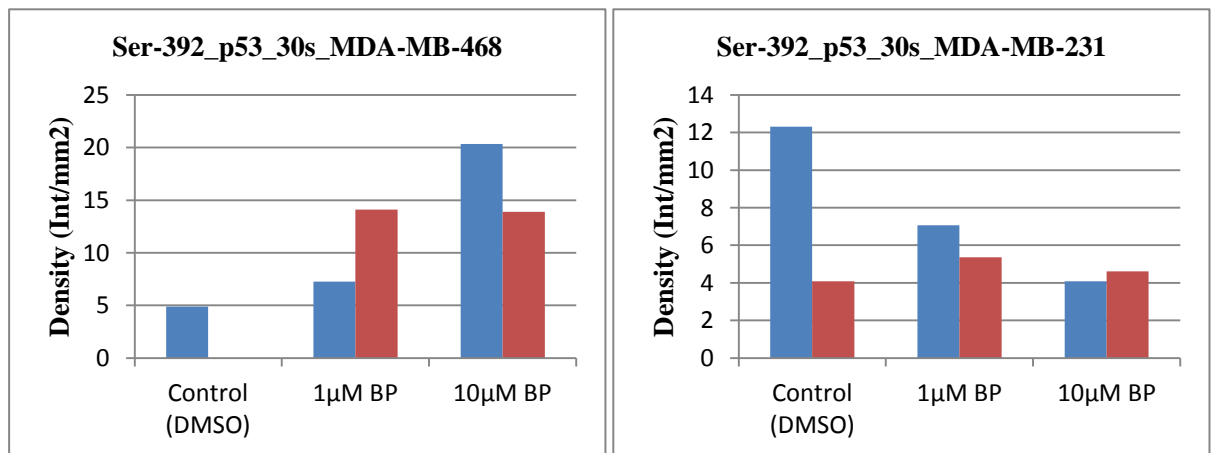
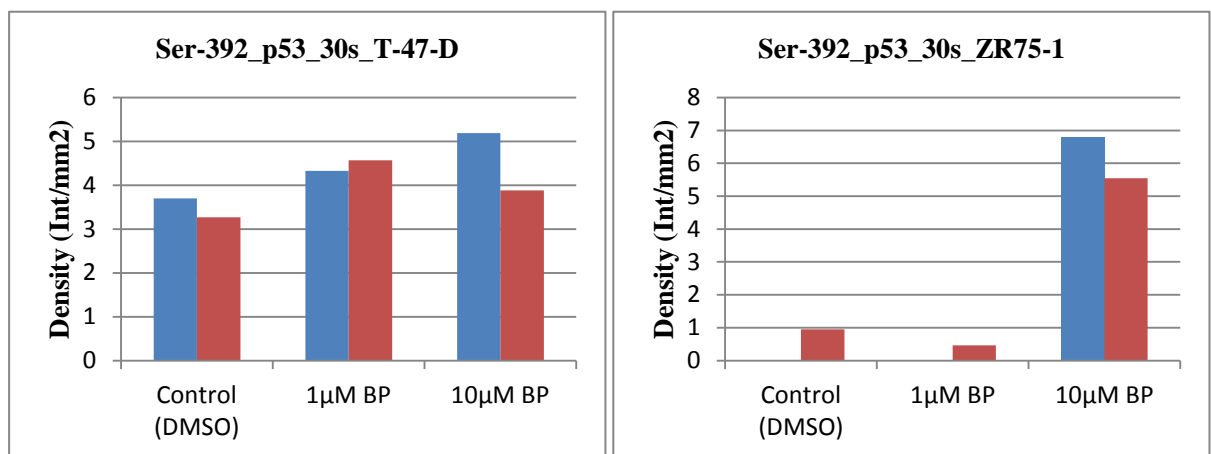
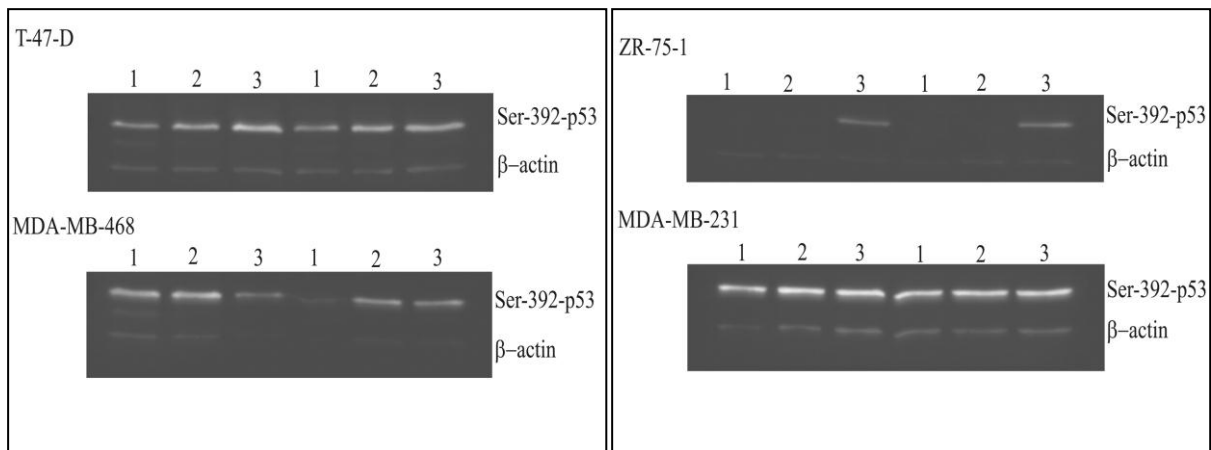


Figure 15. Immunoblotting of phosphoSer392-p53 protein gained from whole cell protein fractions (10-20 μg) of various breast cancer cells. Cells were exposed to 1 μM and 10 μM BP in duplicates for 48h. Numbers describe (1) control (2) 1 μM BP (3) 10 μM BP. Bar charts are drawn from densitometry values of immunoblotting results. Results are shown as the ratio of densitometry values of p53 protein and corresponding loading control. Duplicate results are shown as blue and red colors.

6 DISCUSSION

6.1 Benzo(a)pyrene Exposure

BP is well recognized environmental pollutant and is present in significant amounts in tobacco smoke, petroleum products, asphalt and coal-processing waste products. It has been indicated that BP evokes many adverse effects like immunosuppression, teratogenicity and tumor formation after its exposure (Verma et al., 2012). It is known that BP is not a direct carcinogen; instead it is first metabolized by CYP1A1, CYP1B1 and EH to BPDE which is a reactive carcinogen. Huovinen et al (2011) conducted a study on various breast cancer cell lines (MDA-MB231, MDA-MB468, ZR-75-1 and T-47-D) and showed that ZR-75-1, MDA-MB-468 and T-47-D cell lines were capable of metabolizing BP to BPDE (Huovinen et al., 2011). Exposure to BP may increase the risk of breast cancer due to the expression of CYP1A1 and CYP1B1 enzymes in breast tissue.

Several types of PAHs have been discovered which when taken together as mixture had affected toxicity of carcinogenic PAHs by altering their metabolic rate (Rendic and Guengerich, 2012). BP is also assumed to provoke its own metabolism (Donauer et al., 2012). Dose selection is a significant parameter when performing a study on chemical carcinogenesis. In this study two dose levels were used (1 μ M and 10 μ M). In reality exposed dose is much lower than experimental dose. This is because exposure duration in experimental conditions like 48h in this study is shorter than in real life exposures (Lee and Shim, 2007).

As expected, p53 protein level was multiplied in ZR-75-1 cell line containing wt TP53 gene after exposure to BP. However, the cell lines like MDA-MB-468 and T-47-D contained mutated TP53 gene also showed an increase in total p53 protein after BP exposure. The possible explanation might be the inability of mutated p53 to activate MDM2 protein which is involved in negative regulation of p53 protein (Iwakuma and Lozano, 2003, Michael and Oren, 2003, Hainaut and Vahakangas, 1997). The other possible explanation could be that phosphorylation of MDM2 binding site inhibited p53 degradation leading to its accumulation in cells. (Lavin and Gueven, 2006). In this study, p53 protein was not induced by BP in MDA-MB-231 cell line which perhaps related to mutated TP53 gene (Huovinen et al., 2011). Different types of genotoxic stresses commence signaling pathways that temporarily stabilize the p53 protein, induce it to accumulate in the nucleus and activate it as a transcription factor

(Appella and Anderson, 2001, Liu et al., 2008, Cox and Meek, 2010). Various studies conducted In vivo on animal tissues (Serpi et al., 1999) and In vitro on cell lines concluded the induction of p53 protein by BP (Topinka et al., 2008).

6.2 Usage of Breast Cancer Cell Lines

The breast cancer cell lines used in this study possess both benefits and drawbacks (Burdall et al., 2003). Benefits of using cancer cell lines comprise of their ease of handling, relatively high degree of homogeneity, unlimited replication and easy to culture them from frozen stock. The drawbacks existed include that they have tendency to genotypic and phenotypic drift during culture. Variations in growth rate of cells and changes in hormone receptor are also observed (Burdall et al., 2003). The first breast cancer cell line was established in 1958 and after that many of them have been utilized for research purposes. A few of previously described breast cell lines are in current use like MDA-MB-231, T-47-D and MCF-7 (Lacroix and Leclercq, 2004). The description of cancer by use of breast cancer cell lines is still debatable, since most of used cell lines were isolated from metastatic cancer but not from primary tumour (Burdall et al., 2003, Lacroix and Leclercq, 2004). The genetic instability in cancer cell lines may have generated many subpopulations in different laboratories which caused phenotypic changes by time in a specific clone (Burdall et al., 2003, Lacroix and Leclercq, 2004). It is well known that distinct breast cancer cell lines show distinct phenotypes. In this study four different breast cancer cell lines were used to investigate whether the responses of BP exposure are particular to certain cell line or specific to breast tissue. ZR-75-1 cell line has wild type TP53 gene (Engel and Young, 1978), so it can be used as reference model when comparing of BP induced responses in other breast cancer cell lines with mutated TP53 like MDA-MB-231, MDA-MB-468 and T47-D (Huovinen et al., 2011).

6.3 Activation of p53 protein after BP Exposure

Post-translational modification is required for activation of p53 protein (Unger et al., 1999). In this study, phosphorylation of p53 protein at Ser-15 occurred in at some level in all studied cell lines after exposure to BP for 48h, but lowest phosphorylation level was observed in ZR-75-1 cells (wt p53 protein). Because phosphorylation of Ser-15 in p53 is known to prevent the interaction with MDM2, a negative regulator of p53, this results in stabilization of p53 and can increase the amount of p53 protein. However, in this study, p53 induction was clearly observed only in ZR75-1 and MD-MB-468 cells. On the other hand, phosphorylation

of Ser-392 in ZR-75-1 cells containing wt p53 protein was comparatively more prominent than phosphorylation of Ser-15 after BP exposure. This was regarded as first stabilizing event after exposure to BP (Huovinen et al., 2011).

Earlier studies provide contradictory results about phosphorylation of Ser at 392. It was proposed that normal activation of p53 in regulation of cell growth or transcription activation did not require phosphorylation of Ser at 392 (Fiscella et al., 1994). The contradictory claim was that phosphorylation of p53 at Ser392 produced p53 stabilization (Sakaguchi et al., 1997). It is now believed that significant modification in p53 after several stresses causes phosphorylation at Ser-392 (Cox and Meek, 2010). Cox and Meek proposed that phosphorylation of p53 at Ser-392 had significant role in stabilization of p53 protein. Studies conducted on mutated p53 also provide contradictory results (Matsumoto et al., 2006). It was propose that phosphorylation of mutated p53 protein had significant role in cancer development. Moreover, phosphorylation of wt p53 and mutated p53 protein is also modified during tumor development (Matsumoto et al., 2006). In this study, higher level of phosphorylation at Ser-392 was found in all mutated cells in both control and BP treated samples. Higher increase was seen in Ser-392 phosphorylation in MDA-MB-468 cells than ZR-75-1 cells having wt p53 protein. The findings of this study are in accordance with the finding by Matsumoto et al (2006), who proposed that phosphorylation of mutated p53 protein at Ser-392 contribute to carcinogenesis.

6.4 Deliberations for Improving Western Blotting Analysis

Western blotting has been used for detection of protein about 30 years. Its protocol has been modified for many times according to requirements. Many technologies have emerged for optimizing its sensitivity and speed (MacPhee, 2010). Western Blotting propose following *advantages*: (a) wet membranes are flexible and can be handle without difficulty, (b) immobilized proteins on membranes are easily approached by different ligands, (c) less amount of reagents is needed for transfer analysis, (d) more than one duplicates of gel are possible, (e) it as also possible to store transfer pattern for long time, (f) multiple successive analysis can be performed on same transfer protein (Kurien and Scofield, 2006). Besides its advantages, western blotting has considerable *drawbacks* like: (a) amount of total protein consumed per blot is high; (b) this method cannot be accommodated effectively to multiplexed format, (c) nonlinear gel runs and irregular blotting conditions affect quantity of protein transferred to membrane (Loebke et al., 2007). MacPhee (2010) proposed very good

optimization techniques for improving western blotting. Cell lysis process can be improved by optimizing the lysis buffer and by monitoring extraction of protein of interest carefully. Electrophoresis can be made more efficient by optimizing percentages of polyacrylamide, bisacrylamide and acrylamide and by monitoring the pH of buffers used in gel preparation and electrophoresis. Transfer (blotting) can be maximized by selecting best membrane support for analysis on basis of appropriate pore size, binding capacity, durability, effectiveness with ECL and background fluorescence. Detection of proteins can be made more accurate by optimizing dilutions of primary and secondary antibodies, blocking solutions, number of washes in buffer or water, temperature for antibody incubation. Other step to optimize detection process includes selection of appropriate kit for particular analysis (MacPhee, 2010).

7 CONCLUSIONS

Human breast cancer cell lines were used for studying effect of BP on p53 protein which is protective protein e.g. against carcinogenesis. In vivo experiments give also information about effects of BP; however In vitro studies conducted in human breast cancer cell lines can supplement the in vivo results by providing mechanistic information. Various cell lines were used in this study to make the results more meaningful. Among all used cell lines ZR-75-1 cell line was only cell line containing wt TP53 gene (Huovinen et al., 2011). Post-translational modifications of p53 protein were studied and it was noted that BP exposure caused phosphorylation of p53 protein at Ser-392 which stabilized p53 protein in ZR-75-1 cell line. In MDA-MB-468 BP caused clearly Ser-392 phosphorylation while in MDA-MB-231 and T-47-D cells not. It can be concluded from this study that p53 protein was phosphorylated at Ser-392 after BP exposure in both mutated and wt TP53 gene. This study clearly demonstrates the role of omnipresent environmental contaminant like PAHs in human breast cancer etiology. When comparing the amounts of p53 protein in both mutated and wt cells, it is clear that wt cells shows less increase in phosphorylation by BP as compared to mutated ones at Ser-15 level. Phosphorylation in MDA-MB-468 cell line was higher after BP exposure than in ZR-75-1 cell line.

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